Apoptosis regulators as a target for induction of apoptosis of Imatinib resistant chronic myeloid leukemia cells

Tamalika Paul

Registration Number: R-17RS18210108

Department of LIFE SCIENCE

Presidency University

Name of Supervisor: Dr. Nabendu Biswas

Designation: Assistant Professor

Name: Tamalika Paul

Supervisor Name: Dr. Nabendu Biswas

Title of Thesis: Apoptosis regulators as a target for induction of apoptosis of Imatinib resistant chronic myeloid leukemia cells

PhD registration Number: R-17RS18210108

PhD registration Date: 14TH August, 2018

Enrolment Number: PU/PHD/BIOS/2017/023

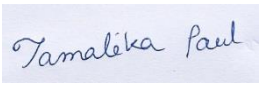
Enrolment Date: 20th September, 2017

Place of Work: Presidency University, Kolkata, West Bengal

Residential Address: 46, Rathtala colony P.O: Ranaghat, Dist: Nadia Pin code:741201

Email id:tamalika_paul@yahoo.com/tamalikapaul3@gmail.com

Phone Number: 9674086951

Signature of Research Scholar: 

Signature of Supervisor:

Contents:

Subject	**Page number**
Abstract	3
Introduction	3-4
Objectives	5
Methodology	5-7
Objective I	
Result	8-19
Objective II	
Result	20-36
Objective III	
Result	39-49
Discussion	49-50
Therapeutical implication	50
Future Direction	51
Reference	51-54
Plagiarism Certificate	
First page of published manuscript	
Certificate of conferences attended	

Apoptosis regulators as a target for induction of apoptosis of Imatinib resistant chronic myeloid leukemia cells

Abstract: Apoptosis is a regulated cell death characterized by cell shrinkage, nuclear condensation, DNA fragmentation, membrane blebbing. There are several apoptosis regulators eg: BCL2, BclXL, BAX, Survivin, FLIP, XIAP, cIAP etc. Targeting these apoptosis regulators may be a viable strategy for the treatment of cancer because one of the hallmarks of cancer is the deregulation of apoptosis. In our first study we were trying to kill chronic myelogenous Leukemia cell line K562, which were resistant to Imatinib, by making them sensitive to TRAIL, a well-studied anti-cancer agent. In previous study, a known anticancer agent, Hydroxychavicol, has been shown to have cytotoxic effect against K562 cells which were sensitive to Imatinib. So, we tested this compound to imatinib resistant K562 cells. In imatinib resistant K562 cells, XIAP, FLIP have emerged as targets by hydroxychavicol to sensitize cells to TRAIL mediated apoptosis via ROS, especially H_2O_2 dependant manner. ROS decreases XIAP, FLIP in imatinib sensitive K562(S) and imatinib resistant K562(R) both. In K562(R) cells, JNK and ERK differentially regulate FLIP and XIAP, respectively. ROS mediated activated ERK decreases Akt phosphorylation, Akt binding to XIAP and stabilization of XIAP. ROS activated JNK increases ITCH expression thereby ITCH binds to FLIP and cause FLIP degradation. So, ROS degrades FLIP, XIAP by ubiquitin mediated pathway. But degradation pathway of these antiapoptotic proteins has identified different in K562(S) cells. In K562(S) cells, ROS degrades XIAP, FLIP by lysosomal degradation pathway. ROS activated JNK causes lysosomal degradation of XIAP, FLIP both. In K562(S) cells, ERK remained activated that cause Akt phosphorylation that increase XIAP, FLIP upregulation in absence of ROS. Activated ERK give antiapoptotic signaling in K562(S) cells. ROS treated K562(S) cells, ERK is dephosphorylated so Akt remains dephosphorylate so, XIAP, FLIP lysosomal degradation is occurred. Thus our findings may enlighten a differential signalling pathway between Imatinib resistant and sensitive cells which may be targeted for the treatment of later stages of the disease of CML patients.

Introduction: Chronic Myeloid leukemia is diseases of myeloproliferative disorder of pluripotent stem cells which results from reciprocal translocation of t(9;22)(q34;q11) and formation of constitutively active Philadelphia Chromosome . In Philadelphia chromosome, fused BCR-ABL gene encodes constitutively active tyrosine kinase protein, BCR-ABL [1].

Imatinib is potent inhibitor of tyrosine kinase ABL, is first-line drug for the treatment of CML [2]. Generation of resistance against imatinib is occurred due to BCR/ABL amplification and over-expression, BCR/ABL kinase domain mutations and clonal evolution with activation of additional oncogenic pathways.

Apoptosis is programme cell death. Apoptosis is important for elimination of infected or damaged cells that interfere with a normal function. The loss of apoptotic control contributes cancer cells to be immortal [3-5]. Regulation of apoptosis, both Intrinsic and Extrinsic, occurs at various levels. FLIP and XIAP are two of the well-established regulators of apoptosis.

FLIP is a pro-caspase 8 mimetic. It contains two DEDs like the initiator caspase. It is anti-apoptotic protein due to the absence of caspase like activity, due to the lack of the catalytic cysteine residue that is present in pro-caspase 8 [6-9]. FLIP competes with caspase-8 to prevent BID activation and inhibits intrinsic apoptotic pathway. FLIP upregulation also blocks TRAIL mediated apoptosis because the recruitment of FLIP to the DISC instead of procaspase-8 or -10 can block caspase activation [10].

XIAP belongs to the Inhibitor of Apoptosis Protein (IAP) family. It contains 1-3 baculovirus IAP repeat (BIR) domains, a carboxyterminal RING (really interesting new gene) zinc-finger domain which possess E3 ubiquitin ligase activity[11,12]. It inhibits active caspase 9 through its BIR3 domain and inhibits active caspase 3,7 through its linker BIR2 domain. XIAP has ubiquitin ligase activity, ubiquitinates the caspase 3 and degrade by proteosome [13-16].

XIAP and FLIP are promising targets for therapeutic involution because these proteins are overexpressed in many cancer cells including haematological malignancy and linked with treatment failure [17,18]. Elevated expression of these antiapoptotic proteins resists agonist antibodies that stimulate Death Receptor (DR) mediated apoptosis [19]. Overexpression of XIAP, FLIP activate different type of signal transduction pathways that enhance cell proliferation, promote cell survival [20,21]. These two actions promote unrestrained tumour growth, resilience that make XIAP, FLIP, promising target for killing cancer cells.

Objectives of my Thesis work:

1. Identification of Inhibitor of apoptotic protein as a target of induction of apoptosis in imatinib resistant CML cells.

2. To identify the modifications of IAPs and signalling mechanisms involved in the modification
3. To check the key findings in imatinib resistant cells from objective I and objective II in imatinib sensitive cells.

Material and Method

Materials: Hydrogen peroxide was purchased from Merck Life Science Pvt. Ltd . Propidium iodide, Imatinib mesylate were bought from Sigma-Aldrich and DR5 blocking chimera rhTRAIL was bought from R&D Systems Inc. (Minneapolis, Minnesota, USA). MTT and NAC (N-acetyl-L-cysteine), 2′,7′- Dichlorofluorescin diacetate were bought from Calbiochem. iScript™ Reverse Transcription Supermix ,SsoFast™ Evagreen® Supermix were purchased from BIO RAD (USA). FITC Annexin V was taken from BD Pharmingen. The antibodies against caspase-8, caspase-9, caspase-3, PARP, DR5, XIAP, c-FLIP, Bcl2, survivin, CIAP, BAX, tBID, Akt, pAkt, PI3K, pPI3K, ERK and JNK, pJNK pERK1/2, ITCH, anti-mouse IgG HRP-linked antibody and anti-Rabbit IgG HRP-linked antibody, Control siRNA, ERK1/2 siRNA, JNK siRNA were bought from Cell Signaling Technology (Denver, Massachusetts,USA). Anti-γ-actin antibody used as a control was bought from Biobharati Life Science (India). pcDNA3 Flag JNK2a1 was a gift from Roget Davis (Addgene plasmid # 13754; http://n2t.net/addgene:13754; RRID: Addgene_13754) and pcDNA3-HA-ERK2 WT was a gift from John Blenis (Addgene plasmid # 8974; http://n2t.net/addgene:8974; RRID: Addgene_8974). SureBeads™ Protein G Magnetic Beads was bought from BIO RAD (USA). JNK inhibitor SP600125, ERK inhibitor PD98059, p38 inhibitor SB203580 were purchased from Abcam (Cambridge, MA, USA). Fetal Bovine Serum (FBS) Standard was purchased from Gibco®.Life Technologies, USA.

Methods:

Cell lines and culture condition: Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum and 0.1% ampicillin and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with continuous flow of 5% carbon dioxide (CO2).

Development of imatinib-resistant K562 cells: Imatinib was added to the cell culture medium in a graded increasing dose, and the viable cells were selected and placed in the medium with next higher doses of imatinib. In this way, final imatinib concentration was

reached at 1.5 μg/ml, where 100% cells were made viable. These cells were designated as K562(R).

Annexin V/PI binding assay: 1.0×10^5 cells were washed with PBS twice and annexin V binding buffer was added into it. Then annexin V/FITC was added in it and kept for 30 mins at dark place. Next 50μg/ml PI was added and flow cytometry was done mixing the cells with 400μl of 1X Annexin V binding buffer. Analysis was done in a flowcytometer (BD accurie) and BD C6 software.

Real time PCR: Using Trizol reagent the total RNA was isolated from appropriately treated cells according to the manufacturer's instructions. cDNA was prepared from isolated total RNA using iScriptTM Reverse Transcription Supermix for RT-qPCR (BIO RAD). Real-time PCR was done with SsoFastTM Evagreen Supermix (BIO RAD). 40 cycles were run for each round of PCR for total saturation and the Cq values were considered for further analysis. All RT-qPCR for complete experiments were independently repeated at least three times. For graphical representation of the gene expression data, Actin was used as house-keeping gene and the mRNA levels were normalized accordingly.

Western Blot: Cells were washed in ice-cold PBS and lysed with 1X RIPA (CST, Cat no 9806S) and protease inhibitor cocktails. Lysate are collected by centrifugation at 15000 rpm for 15 min. Proteins samples were boiled with Laemmli buffer at 95 °C for three minutes and subjected to SDS PAGE. Then Proteins were transferred to PVDF membrane. Then the membranes were blocked with 5% BSA (for phospho-epitope) or 5% not fat milk diluted in TBST for 1 hr. Proteins were detected using the respective antibodies corresponding HRP linked secondary antibodies were used for detection. Bands were developed using BIO RAD Clarity™ Western ECL substrate. Image were developed by BIO RAD ChemiDoc™ MP Imaging System.

Knock down assay: 2×10^6 cells/well were kept in overnight without serum and antibiotics and transfected with siRNA oligo using Lipofectamine 2000 (Invitrogen) and RPMI (without serum and antibiotic) medium, according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose–response studies. Silencing was examined 48 h after transfection.

Gene Overexpression: To ectopically overexpress JNK and ERK gene, cells were transfected with pcDNA3 Flag JNK2a1 (Addgene plasmid # 13754) and pcDNA3-HA-ERK2 WT (Addgene plasmid # 8974) using Lipofectamine 2000 according to the manufacturer's protocol. Cells were treated with H_2O_2 24 hr post transfection and overexpression was checked by western blot.

Objective I: Identification of Inhibitor of apoptotic protein as a target of induction of apoptosis in imatinib resistant CML cells.

Imatinib is widely accepted drug for the treatment of CML. Development of resistance of this drug is major problem for the treatment of CML. Alternative therapeutic approach might prove effective treatment for the imatinib resistance patients with CML.TRAIL (The tumor necrosis factor-related apoptosis-inducing ligand) could be this alternative treatment choice because TRAIL acts as promising anticancer drug as TRAIL shows high toxicity toward cancer cells and less toxicity toward the normal cells [22]. However K562(R) cells are TRAIL resistant. Combination therapeutic approach involving TRAIL along with any agent that induces K562(R) to TRAIL mediated apoptosis that could be an interesting concept in the treatment of imatinib resistant CML patients [23]. In this objective we use Hydroxychavicol which is a phenolic compound of Piper betel leaves. Previous study suggested that Hydroxychavicol has anticarcinogenic activity against CML [24,25,26].

Hydroxychavicol sensitizes K562(S) cells to drug induced apoptosis. We use Hydroxychavicol to extend that work into resistant cells. TRAIL has been shown to induce Extrinsic apoptotic pathway (27-32). However, K562(R) cells were not sensitive to TRAIL.Therefore; we use Hydroxychavicol to sensitize K562(R) cells to TRAIL induced apoptosis.

Result:

Hydroxychavicol sensitizes imatinib-resistant CML cell lines to TRAIL-induced apoptosis

To check resistance of this K562 cells to imatinib, K562 cells were treated 1.5 µg/ml of Imatinib. Annexin V/PI binding assay showed that imatinib induced almost 37% cell death in normal untreated K562 cells whereas only 10% cell death was observed in case of the imatinib-resistant K562(R) cell (Fig1A).

When two cell lines (both imatinib sensitive and imatinib resistant) were incubated with various doses of TRAIL, annexin V/PI binding assay showed very little or no cell death in K562(R) cells by TRAIL (Fig1B). Imatinib resistant K562 cells are used in subsequent experiments.

To confirm role of Hydroxychavicol on TRAIL mediated apoptosis, K562 (R) cells were treated with Hydroxychavicol for 6 hrs. , then incubated with 200ng/ml TRAIL for 18 hrs. Annexin V/PI binding assay and MTT assay confirmed that Hydroxychavicol and TRAIL alone induced only 5–10% cell death. Cytotoxicity increased significantly after 24 hrs when both were used in combination (Fig 1C,1D). Next, caspase cleavage assay was done to confirm apoptosis enhancement by TRAIL. K562(R) cells were treated with 4 μmol/l and 6 μmol/l of Hydroxychavicol alone or in combination of 200 ng/ml TRAIL for 18 h. Result showed that combinatory treatment significantly enhanced cleavage of caspase 3,8, PARP, no caspase 9 cleavage is occurred. Pro caspase 8 cleavage is occurred, It suggested that extrinsic pathway is involved in HCH mediated TRAIL induced apoptosis (Fig1e).

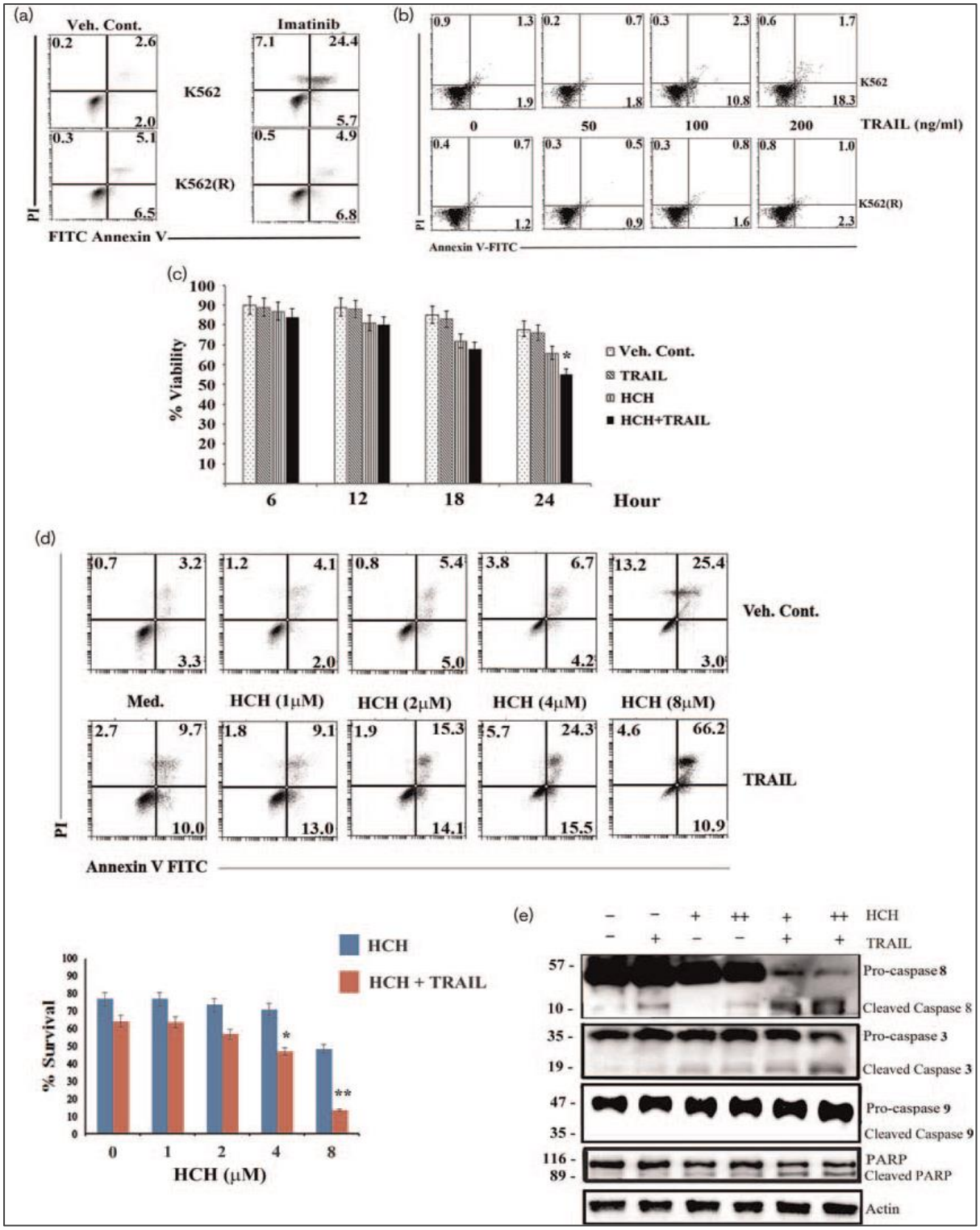

(a)
Veh. Cont.
Imatinib
K562
K562(R)
PI
FITC Annexin V
(b)
K562
K562(R)
TRAIL (ng/ml)
0
50
100
200
PI
Annexin V-FITC
(c)
% Viability
Veh. Cont.
TRAIL
HCH
HCH+TRAIL
Hour
(d)
Veh. Cont.
TRAIL
Med.
HCH (1μM)
HCH (2μM)
HCH (4μM)
HCH (8μM)
PI
Annexin V FITC
(e)
HCH
TRAIL
Pro-caspase 8
Cleaved Caspase 8
Pro-caspase 3
Cleaved Caspase 3
Pro-caspase 9
Cleaved Caspase 9
PARP
Cleaved PARP
Actin
% Survival
HCH
HCH + TRAIL
HCH (μM)

Figure 1

Fig1: Hydroxychavicol enhances the sensitivity of imatinib-resistant K562(R) cells to TRAIL. a) Imatinib sensitive K562 and Imatinib resistant K562 (R) cells were treated with 1.5µg/ml imatinib. Cells were analysed by annexinV/PI binding assay by flow cytometry b) K562(R) and sensitive K562 cell were treated with indicated doses of TRAIL, and annexin V/PI binding assay was performed in flowcytometer. C) K562(R) cells were treated with 6µmol/l of HCH and 200 ng/ml of TRAIL. After 24 hrs, cell viability was measured by MTT assay. Data represent mean ±SD of three independent experiments (*P<0.05). d) K562(R) cells were treated with indicated doses of HCH and 200 ng/ml of TRAIL. Annexin V/PI binding assay was performed in flowcytometer. Data represent mean ±SD of three independent experiments (*P<0.05). e) K562 (R) cells were treated with Hydroxychavicol and TRAIL (tumour necrosis factor-related apoptosis-inducing ligand). Whole cells lysate were subjected for Western blot analysis using antibody against pro caspases-8, 3, 9 and PARP, and actin was used as a loading control.

Altered signaling downstream to TRAIL receptors DR4 and DR5 plays a key role in Hydroxychavicol-mediated TRAIL sensitization in K562(R) cells:

As TRAIL binds to death receptor and induces apoptosis. We wanted to confirm the involvement of TRAIL-apoptosis pathway by Hydroxychavicol and TRAIL. To do that, Cells were preincubated with DR5 blocking chimera antibody for antagonizing TRAIL binding for 2hrs, then treated with Hydroxychavicol and TRAIL either alone or in combination. The flowcytometer analysis showed that DR5 blocking chimera antibody significantly reversed TRAIL-mediated apoptosis (Fig2a). This indicated that TRAIL mediated extrinsic apoptotic pathway was involved in above combinatory effect. One possibility might be that whether Hydroxychavicol increased DR4 and DR5 expression. Cells were treated with Hydroxychavicol at various time points. Result indicated that there was no increase in DR4 and DR5 at 18 hr (Fig2b). Data indicated that DR4, DR5 remains unaltered at various doses of Hydroxychavicol (Fig2c). DR4, DR5 expressions at mRNA level remain unaltered at various doses of HCH (Fig2d). DCR1, DCR2 are both TRAIL receptors. But DCR1/DCR2 cannot induce apoptosis [33,34]. There was no change in DCR1/DCR2 at protein level (Fig2e) and mRNA level (Fig2f) after treatment of Hydroxychavicol. Our data indicated that Hydroxychavicol involved death receptor-mediated signaling pathway for induction TRAIL mediated apoptosis, while death receptor expression remains unaltered.

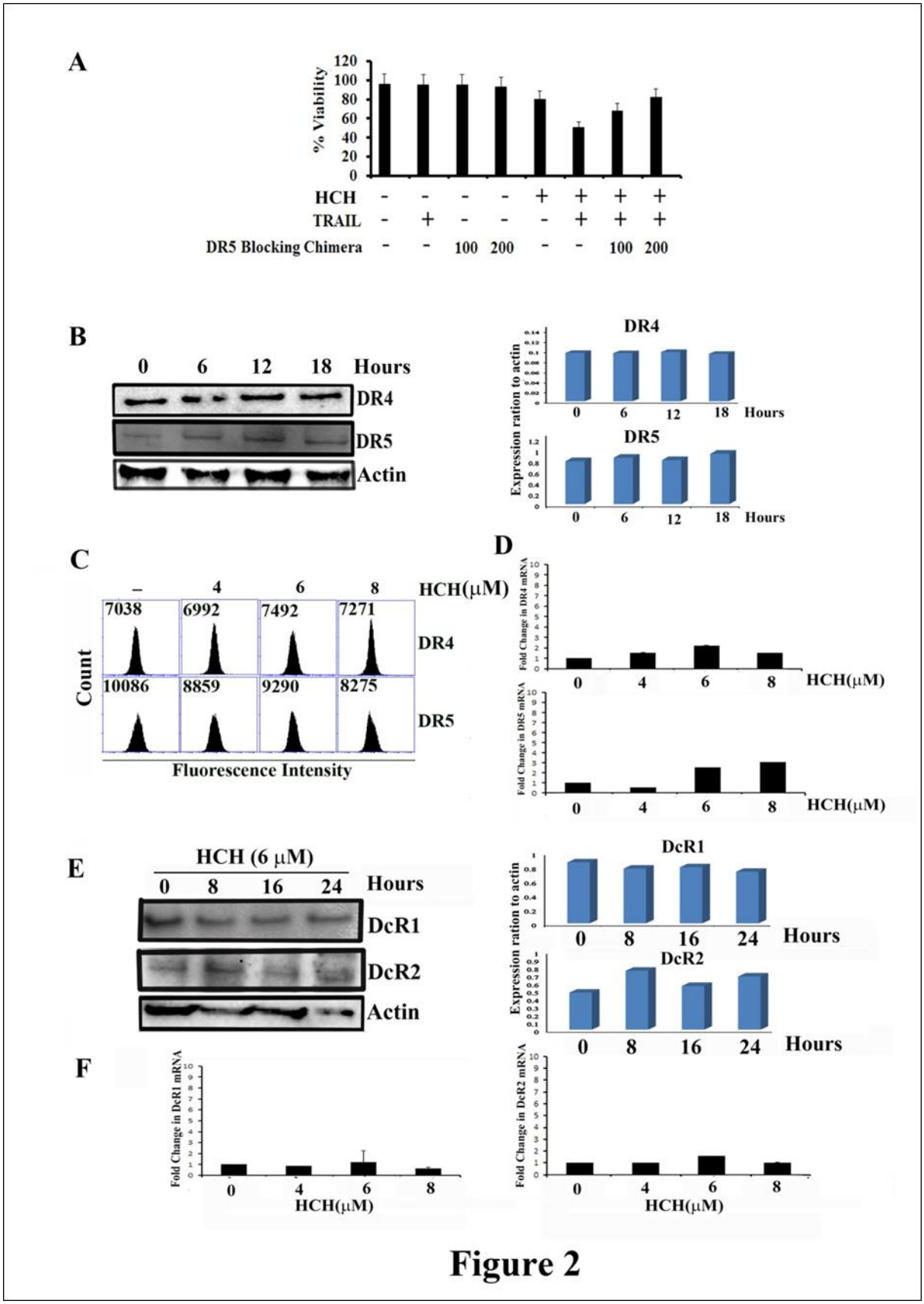

A
% Viability
120
100
80
60
40
20
0
HCH - - - - + + + +
TRAIL - + - - - + + +
DR5 Blocking Chimera - - 100 200 - - 100 200
B
0 6 12 18 Hours
DR4
DR5
Actin
DR4
Expression ration to actin
0 6 12 18 Hours
DR5
0 6 12 18 Hours
C
– 4 6 8 HCH(μM)
7038 6992 7492 7271
DR4
10086 8859 9290 8275
DR5
Count
Fluorescence Intensity
D
Fold Change in DR4 mRNA
0 4 6 8 HCH(μM)
Fold Change in DR5 mRNA
0 4 6 8 HCH(μM)
E
HCH (6 μM)
0 8 16 24 Hours
DcR1
DcR2
Actin
DcR1
Expression ration to actin
0 8 16 24 Hours
DcR2
0 8 16 24 Hours
F
Fold Change in DcR1 mRNA
0 4 6 8
HCH(μM)
Fold Change in DcR2 mRNA
0 4 6 8
HCH(μM)

Figure 2

Fig2: Extrinsic apoptotic pathway is involved in TRAIL mediated apoptosis without increase expression of death receptor. (a) K562(R) cells were pretreated with DR5 blocking chimera antibody and then treated with the combination of HCH and TRAIL. Cell viability assay was performed by MTT assay. Data represent mean ±SD of three independent experiments (*P< 0.05). (b) K562(R) cells were treated with various doses of Hydroxychavicol. Whole cell lysate were subjected to western blot analysis for checking DR4/DR5 at protein level. β-actin acts as a loading control. (c) K562 cells were treated with various doses of Hydroxychavicol for 14 hrs. DR4 and DR5 were checked in flowcytometer by labelling them with DR4/DR5 antibody and fluorescent conjugated secondary antibody. Data shown here area representative histogram of three independent experiments. (d) Cells were treated with various doses of Hydroxychavicol. DR4 and DR5 expression were checked by real-time PCR (n =3: data as mean ±SEM). (e) K562 (R) cells were treated Hydroxychavicol for different time point. DcR1 and DcR2 surface protein levels were checked by Western blot analysis. Here β-actin acts as a loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. (f)K562(R) cells were treated with increasing doses of Hydroxychavicol. DcR1 and DcR2 mRNA expressions were checked by real time PCR (n=3,mean ± SD).

HCH decreased FLIP and XIAP at the protein level

Overexpression of antiapoptotic genes are most commonly reported mechanism for apoptosis evasion [35-39]. To check whether any alteration of these proteins could be responsible here. Cells were treated with Hydroxychavicol at different doses at 20 hrs. Result showed that XIAP and FLIP were decreased at protein level. Bcl2 and cIAP levels remains unaltered with increasing doses of HCH (Fig3a). There was no any alternation of FLIP, cIAP, XIAP at mRNA level (Fig3b). To understand the molecular mechanism behind FLIP, XIAP downregulation, cells were treated with MG132 (proteosomal inhibitor) before incubation with Hydroxychavicol, data showed that significant inhibition of FLIP and XIAP downregulation by MG132 (Fig3c). So, XIAP, FLIP degradation is occurred via proteosomal mediated pathway. To check XIAP, FLIP downregulation played a role in apoptosis, cells were knocked down with XIAPsiRNA and FLIPsiRNA before incubation with TRAIL. This result indicated that knocking down of XIAP and FLIP enhanced apoptosis as compared with control siRNA (fig3d).

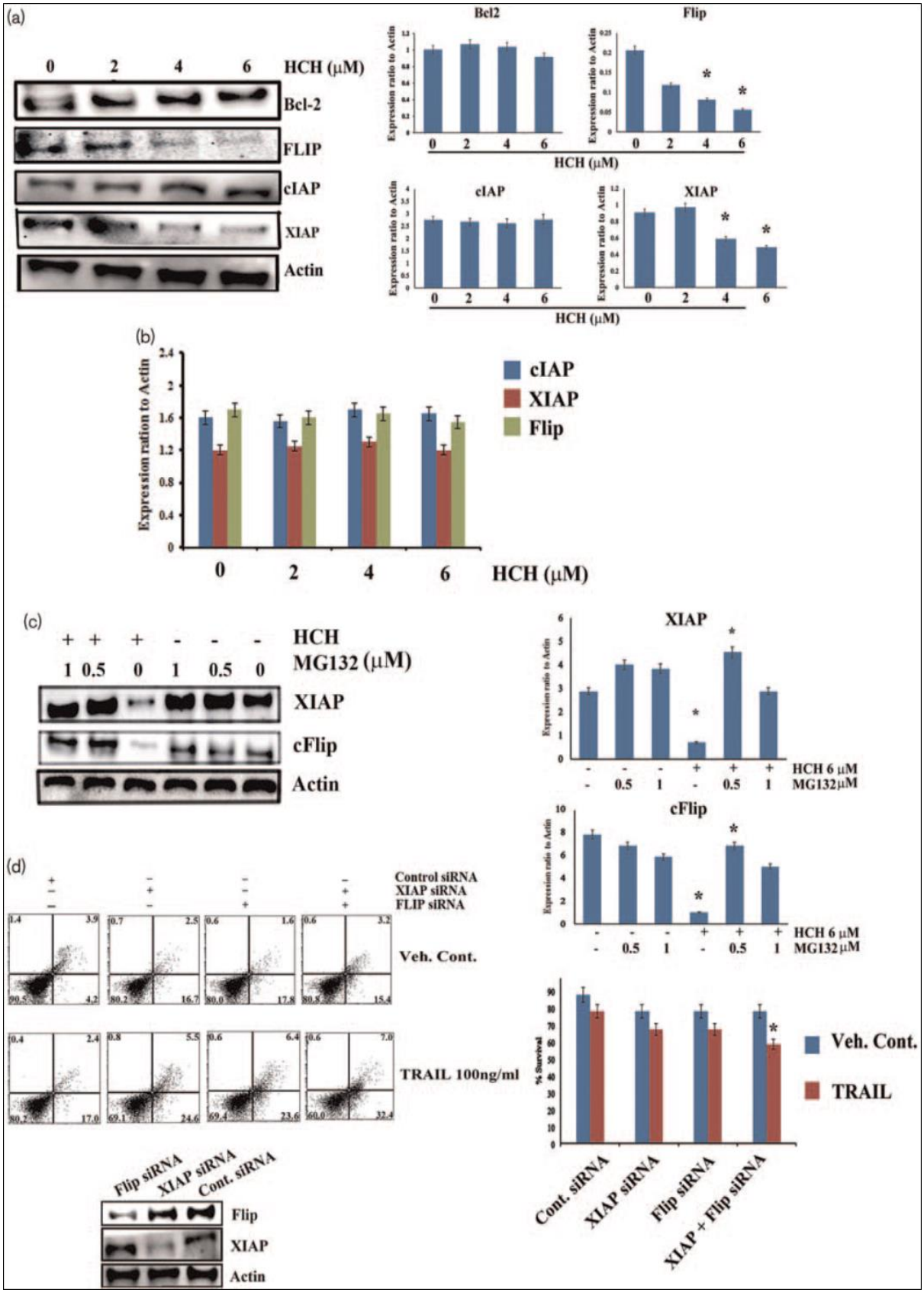

(a)
0 2 4 6 HCH (μM)
Bcl-2
FLIP
cIAP
XIAP
Actin
Bcl2
Flip
cIAP
XIAP
Expression ratio to Actin
HCH (μM)
(b)
Expression ration to Actin
cIAP
XIAP
Flip
HCH (μM)
(c)
HCH
MG132 (μM)
1 0.5 0 1 0.5 0
XIAP
cFlip
Actin
HCH 6 μM
MG132μM
(d)
Control siRNA
XIAP siRNA
FLIP siRNA
Veh. Cont.
TRAIL 100ng/ml
Flip siRNA
XIAP siRNA
Cont. siRNA
Flip
XIAP
Actin
% Survival
Veh. Cont.
TRAIL
Cont. siRNA
XIAP siRNA
Flip siRNA
XIAP + Flip siRNA

Figure 3

Fig 3: XIAP and FLIP downregulation is involved in TRAIL mediated apoptosis. (a) K562 (R) cells were treated with 6 μmol/l HCH for 18 h and whole cell lysates were subjected to Western blot analysis. Data shown here is a representative Western blot of three independent experiments. On the right, data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ±SD of three independent experiments (*P< 0.05). (b) Levels of endogenous c-FLIP, cIAP, and XIAP mRNA were assessed by real-time PCR after treatment with increasing doses of Hydroxychavicol for 14 hrs. and graphically represented as expression ratio to actin as mean ±SD of three independent experiments.(c) K562(R) cells were preincubated with MG132 and then treated with Hydroxychavicol for 16 hrs. Whole cell lysate were subjected to western blot. The Western blot data are a representative one from three independent experiments. Data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments (*P<0.05). (d) K562(R) cells were transfected with control siRNA, FLIPsi RNA, XIAPsi RNA for 48 h and then treated with 200 ng/ml TRAIL. Apoptosis assay was done by annexin V/PI binding assay by flowcytometer. Data represent mean ±SD of three independent experiments (*P<0.05). At bottom panel, XIAP and FLIP level was checked after knockdown of cell with XIAP siRNA and FLIP siRNA respectively.

ROS played a major role in Hydroxychavicol -mediated TRAIL sensitization

It is reported that Hydroxychavicol increases the intracellular ROS level [29,30]. When cells were preincubated with ROS scavenger NAC then treated with Hydroxychavicol and TRAIL, NAC reversed TRAIL mediated apoptosis. This indicated that ROS involvement in this process (Fig4a). ROS generation assay showed that ROS was increased from 15 min, reached peck level at 30 min after treatment of Hydroxychavicol (Fig4b). TRAIL did not change ROS level when TRAIL incubated with various concentrations alone (Fig4c). ROS level was increased at various concentration of Hydroxychavicol, keeping TRAIL concentration fixed (Fig4d). It confirmed that TRAIL has no additive effect in ROS generation.

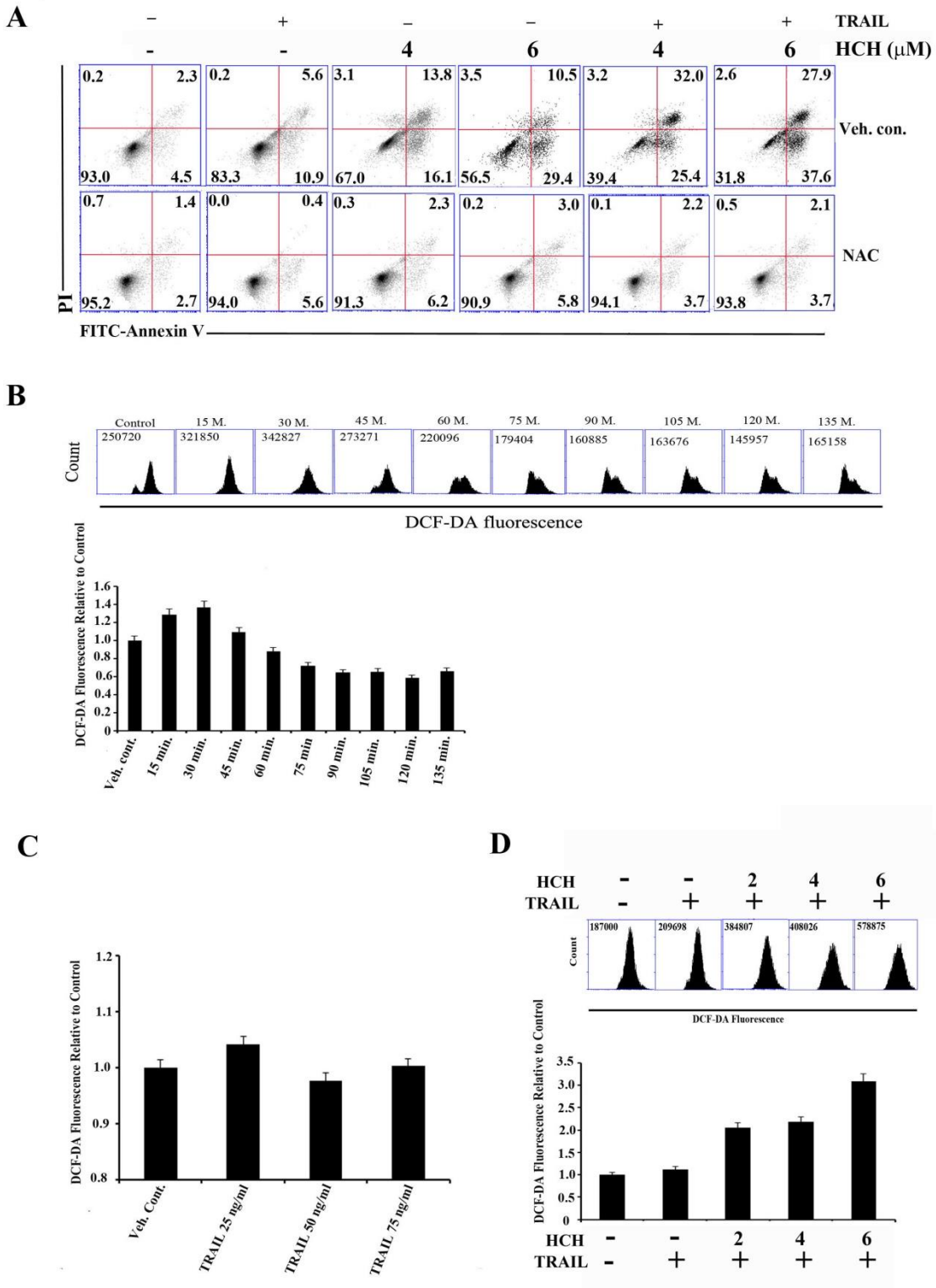

A
TRAIL
HCH (μM)
Veh. con.
NAC
PI
FITC-Annexin V
B
Control
15 M.
30 M.
45 M.
60 M.
75 M.
90 M.
105 M.
120 M.
135 M.
Count
DCF-DA fluorescence
DCF-DA Fluorescence Relative to Control
Veh. cont.
15 min.
30 min.
45 min.
60 min.
75 min
90 min.
105 min.
120 min.
135 min.
C
DCF-DA Fluorescence Relative to Control
Veh. Cont.
TRAIL 25 ng/ml
TRAIL 50 ng/ml
TRAIL 75 ng/ml
D
HCH
TRAIL
Count
DCF-DA Fluorescence
DCF-DA Fluorescence Relative to Control

Figure 4

Fig4: HCH induced TRAIL sensitization of K562(R) cell is ROS dependent: (a) K562 (R) cells were preincubated with ROS scanverger, NAC, and treated with Hydroxychavicol and TRAIL for 24 hrs. Flow cytometry analysis was done after staining with Annexin V and PI . Results were representative of three independent experiments. (b) K562(R) cell line was treated with HCH (6 μmol/l), and then cells were stained with DCFDA for measuring RS level in Flow cytometry. The histograms are representative data of three independent experiments. Below is the bar diagram of mean ± SD of three independent experiments.(c) K562(R) cell line was treated with various doses of TRAIL and ROS level was measured in flowcytometer after incubation with DCFDA. The bar diagram represents mean ±SD of three independent experiments. (d) K562(R) cells were incubated with 200 ng/ml of TRAIL, and different doses of Hydroxychavicol, and DCFDA fluorescence was measured in flowcytometer. Bar diagram of mean ±SD of three independent experiments..

Downregulation of XIAP and FLIP happens in ROS dependant manner

To check whether Hydroxychavicol mediated XIAP, FLIP downregulation was ROS mediated, cells were preincubated with NAC then treated with Hydroxychavicol. Result indicated that NAC reversed HCH mediated XIAP, FLIP downregulation (Fig5a). If ROS have role in XIAP, FLIP downregulation, adding ROS externally should show similar effect. H_2O_2 downregulate XIAP, FLIP after treatment with different doses of H_2O_2.(Fig5b). If ROS is responsible for this downregulation, then ROS itself should sensitize K562(R) cells to TRAIL. TRAIL was applied on H_2O_2-pretreated cells. Data indicated that TRAIL enhanced apoptosis in H_2O_2-treated cells (Fig5c). The role of ROS in Hydroxychavicol-mediated TRAIL-induced apoptosis was confirmed by TRAIL-mediated caspases-8, 3, PARP cleavage which was reversed by NAC (Fig5d).

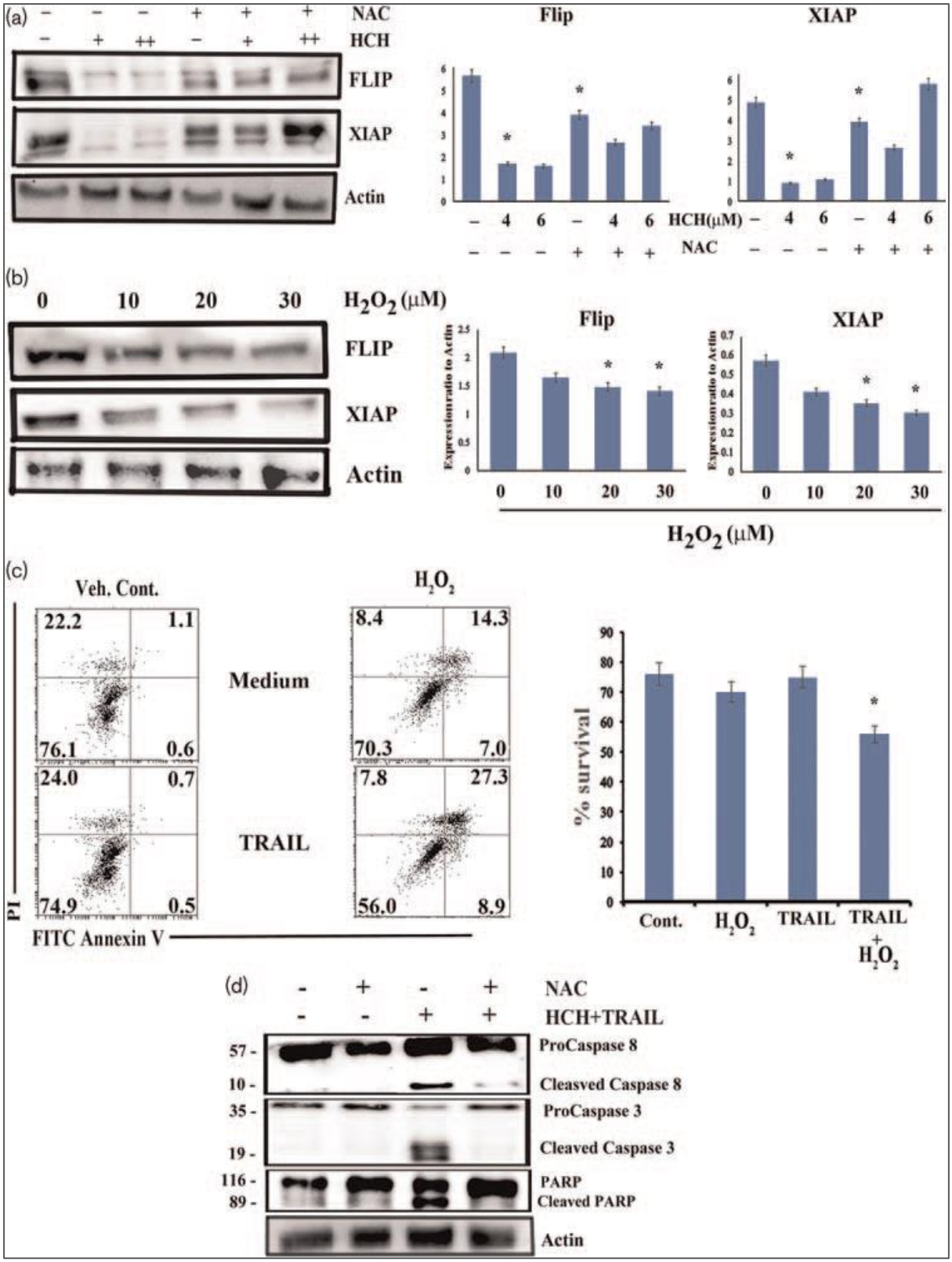

(a)
NAC
HCH
FLIP
XIAP
Actin
Flip
XIAP
HCH(μM)
NAC
(b)
H_2O_2 (μM)
FLIP
XIAP
Actin
Flip
XIAP
Expressionratio to Actin
H_2O_2 (μM)
(c)
Veh. Cont.
H_2O_2
22.2
1.1
76.1
0.6
8.4
14.3
70.3
7.0
Medium
24.0
0.7
74.9
0.5
7.8
27.3
56.0
8.9
TRAIL
PI
FITC Annexin V
% survival
Cont.
H_2O_2
TRAIL
TRAIL + H_2O_2
(d)
NAC
HCH+TRAIL
57 -
10 -
35 -
19 -
116 -
89 -
ProCaspase 8
Cleasved Caspase 8
ProCaspase 3
Cleaved Caspase 3
PARP
Cleaved PARP
Actin

Figure 5

Fig5: ROS plays a role in Hydroxychavicol mediated XIAP, FLIP downregulation: (a) K562(R) cells were preincubated with NAC, then treated with Hydroxychavicol. Whole cell lysate were subjected to western blot analysis to measure XIAP, FLIP protein level. Data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments (*P<0.05). (b) Cells were treated with indicated dose of H_2O_2 for 16 hrs. FLIP and XIAP protein level were checked by western blot. Data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ±SD of three independent experiments (*P<0.05).(c) K562 (R) cells were treated with TRAIL and H_2O_2. Apoptosis assay was performed by annexin V/PI binding assay. Data are a representative dot plot of three independent experiments. The data are also represented as Bar graph which denotes mean ±SD of viable cells [100– (upper left upper right lower right quadrant percent)] from three independent experiments (*P< 0.05). (d) K562(R) cells were preincubated with NAC, and treated with HCH and TRAIL. Whole cell lysate were subjected to western blot analysis for checking the protein level of pro caspase-8, 3, and PARP. Data are a representative blot of three independent experiments.

It is reported that IAPs can be targeted by several agent. From objective I, it is confirm that IAPs can be targeted by Hydroxychavicol thereby TRAIL mediated apoptosis can be induced in imatinib resistant CML cells.

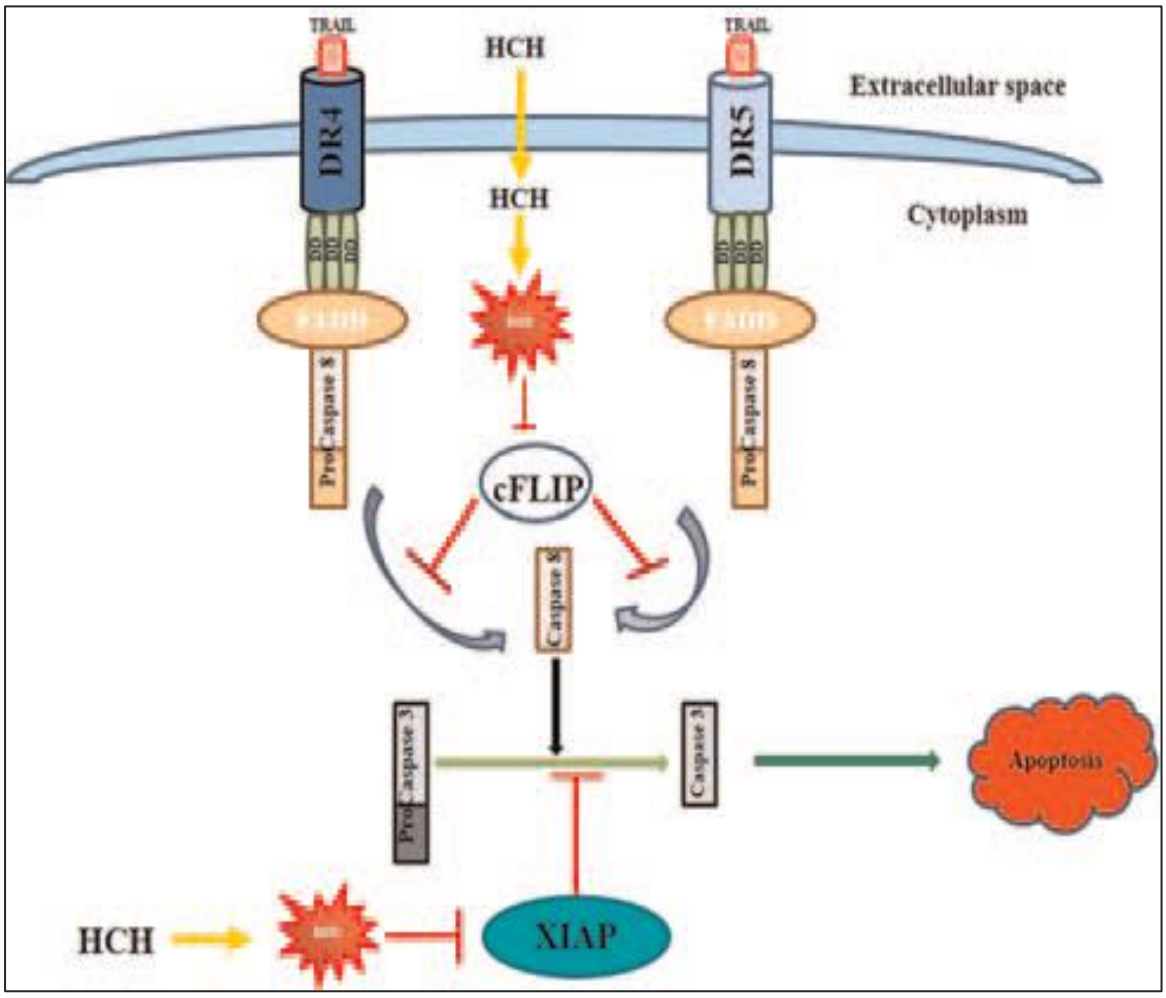


Figure 6: The mechanism of action of HCH in the sensitization of TRAIL in K562(R) cells. HCH sensitizes K562-resistant CML cells to TRAIL-induced apoptosis through downregulation of XIAP and FLIP via ROS generation.

Objective II: To identify the modifications of IAPs and signalling mechanisms involved in the modification

In objective I, we have reported that downregulation of XIAP, FLIP become a strong strategy for TRAIL mediated apoptosis in imatinib resistant CML. Hydroxychavicol downregulate XIAP, FLIP in ROS dependant manner, specifically H_2O_2 dependant manner. In this objective, we have examined that the signalling mechanism that is involved in H_2O_2 dependant XIAP, FLIP downregulation.

Result:

H_2O_2 downregulates XIAP and FLIP:

To check effect of ROS on XIAP, FLIP downregulation, cells were treated with different doses of H_2O_2. Result indicate that H_2O_2 dose dependently decreased FLIP, XIAP at protein level (Fig7a). XIAP, FLIP are decreased at 16 hrs and 12 hrs respectively after treatment cells with H_2O_2 at different time points (Fig7b).As cells were treated with H_2O_2, there might be possible that induction of apoptosis, reduction in cellular protein level nonspecifically. K562(R) cells, treated with different doses of H_2O_2 for 24 hours. Result showed that there was no cytotoxicity after 24 hr (Fig7c). To check RNA level of XIAP, FLIP, cells were treated with various doses of H_2O_2. Real time PCR data indicated that there was no significant increase of XIAP, FLIP at RNA level (Fig7d). When cells were pretreated with NAC followed by H_2O_2, NAC significantly reversed XIAP, FLIP downregulation (Fig7e). So, this confirmed that H_2O_2 downregulates XIAP, FLIP at protein level.

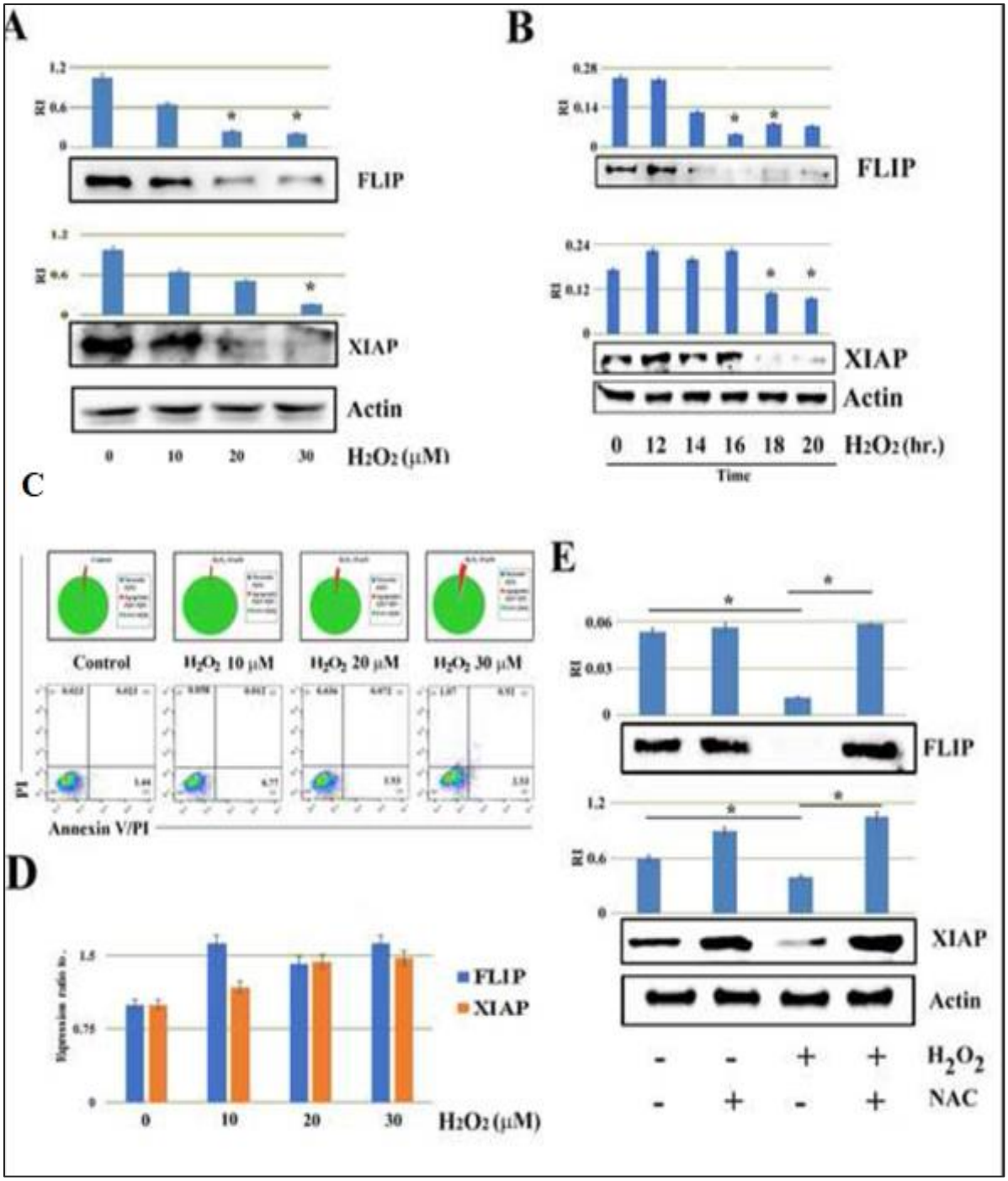

A
RI
FLIP
XIAP
Actin
0 10 20 30 H2O2 (μM)
B
RI
FLIP
XIAP
Actin
0 12 14 16 18 20 H2O2 (hr.)
Time
C
Control
H2O2 10 μM
H2O2 20 μM
H2O2 30 μM
PI
Annexin V/PI
D
Expression ratio to -
FLIP
XIAP
0 10 20 30 H2O2 (μM)
E
RI
FLIP
XIAP
Actin
- - + + H2O2
- + - + NAC

Figure 7

Fig7: ROS downregulates XIAP and FLIP. (a) K562(R) cells were treated with increasing concentration of H_2O_2. Whole cell lysate were subjected to western blot analysis for checking the protein level of XIAP, FLIP. Gamma Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05. (b) K562(R) cells were treated with 30µM H_2O_2 for indicated time points. Western blot analysis of FLIP and XIAP was performed. Gamma Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05. (c) K562(R) cells were treated with indicated doses of H_2O_2 for 18hrs. Cells were subjected to annexin V/ PI binding assay by flow cytometry. Upper panel is the graphical representation of various population in pie chart and lower panel represents representative dot plots. (d) K562(R) cells were treated with increasing concentration of H_2O_2. After 16 hrs, total RNA was extracted from cells. Cellular level of FLIP and XIAP mRNA were checked by Real time PCR. It graphically represented as fold change. Data represent mean ± SD of three independent experiments. (e) K562(R) cells were pre-incubated with 1mM NAC for 1 hr and then treated with 30µM H_2O_2 for overnight. Whole cells lysate were subjected to western blot analysis for checking protein level of XIAP, FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05.

XIAP and cFLIP degradation is proteasomal dependent

Cellular proteins level gets reduced by Lysosomal or Proteosomal degradation pathway. So, Cells were pretreated with 1.5µM proteasome inhibitor MG132, then treated with 30µM H_2O_2. Western blot analysis indicated that MG132 reverses H_2O_2 mediated degradation of XIAP, FLIP (Fig8a,8b upper-panel).Cells were preincubated with MG132 then treated with H_2O_2, cell lysate were taken after 12hrs and 16hrs, subjected with western blot analysis by anti-ubiquitin antibody after immunoprecipitation with FLIP, XIAP antibody. The result showed that MG132 increased XIAP, FLIP ubiquitination by H_2O_2 (FIG8a,8b). To check involvement of lysosomal pathway, cells were preincubated with Chloroquine, then treated with 30µM H_2O_2. Result indicated that Chloroquine didn't reverse H_2O_2 mediated XIAP and FLIP downregulation (Fig8c). These confirmed that H_2O_2 mediated downregulation of FLIP, XIAP is proteosomal mediated.

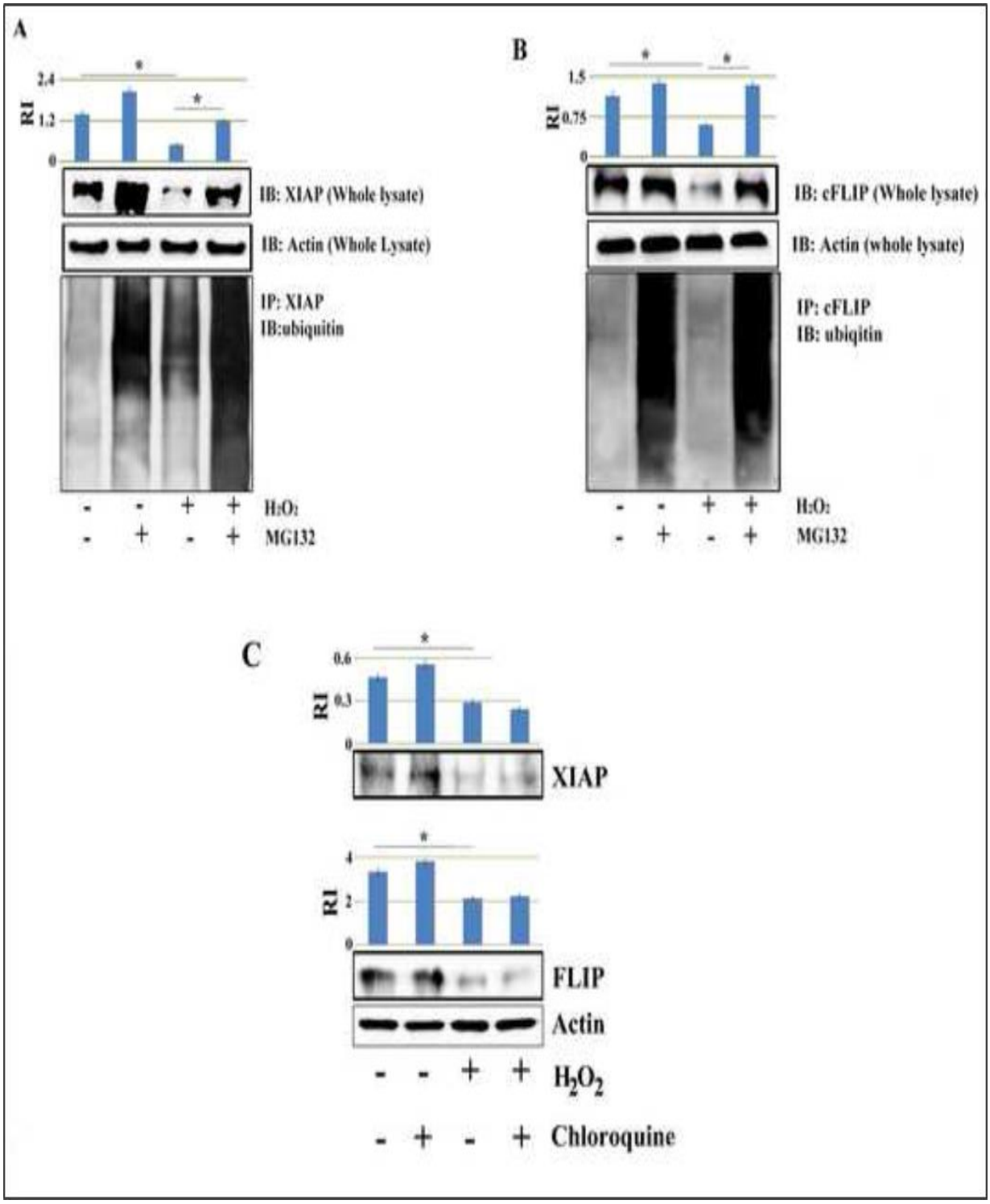

A
RI
2.4
1.2
0
IB: XIAP (Whole lysate)
IB: Actin (Whole Lysate)
IP: XIAP
IB:ubiquitin
H2O2
MG132
B
RI
1.5
0.75
0
IB: cFLIP (Whole lysate)
IB: Actin (whole lysate)
IP: cFLIP
IB: ubiqitin
H2O2
MG132
C
RI
0.6
0.3
0
XIAP
RI
4
2
0
FLIP
Actin
H2O2
Chloroquine

Figure 8

Fig 8: XIAP and cFLIP degradation is proteasomal dependent and does not involve lysosomal degradation (a) K562 (R) cells were preincubated with 2 μM of MG132 and then 30μM H_2O_2. Whole cell lysates were subjected to co-Immunoprecipitation with XIAP antibody and then western blot analysis was performed with anti-ubiquitin antibody. The upper panel shows the whole cell lysates and lower panel show the immunoprecipitates. Polyubiquitination of XIAP was happened in lower panel. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05. (b)) K562 (R) cells were preincubated with 2 μM of MG132 and then 30μM H_2O_2. Whole cell lysates were subjected to co-Immunoprecipitation with FLIP antibody then western blot analysis was performed with anti-ubiquitin antibody. . The upper panel shows the whole cell lysates and lower panel show the immunoprecipitates. Polyubiquitination of FLIP was happened in lower panel Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05. (c) K562(R) cells were preincubated with 20 μM Chloroquine and then treated with 30 μM H_2O_2. Whole cell lysates were subjected to western blot analysis with anti-FLIP and anti-XIAP antibodies. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05.

Activation of ERK and JNK involved in XIAP, FLIP downregulation:

MAPK pathway is major stress signalling pathway. Since ROS downregulates XIAP, FLIP. We checked whether MAPK pathway was involved in this downregulation. Cells were incubated with ERK signalling inhibitor PD98059, then treated with H_2O_2 .Result showed that PD98059 significantly reversed H_2O_2 mediated XIAP down-regulation after 18 hrs. but FLIP was not reversed by PD (Fig9a). Similar experiment was performed with JNK inhibitor SP600125. Result indicated that SP reversed H_2O_2 mediated FLIP down-regulation but not XIAP (Fig9b).Similar experiment was performed with P38 inhibitor SB202190. it didn't reverse either FLIP or XIAP(Fig9c). This confirmed that JNK, ERK were involved in FLIP, XIAP downregulation respectively. JNK, ERK show activity after phosphorylation. Cells were treated with 30 μMH_2O_2 for different time points. Result indicated that JNK phosphorylation increased around 4 hrs and peck around 16 hrs of incubation with H_2O_2. ERK phosphorylation increased around 6 hrs, decreased after 16 hrs

to its basal level (Fig9D, 9E). Protein level of JNK, ERK was unchanged after H_2O_2 treatment (Fig9F). So, H_2O_2 phosphorylate, activate ERK, JNK and these activation involved in XIAP, FLIP downregulation respectively.

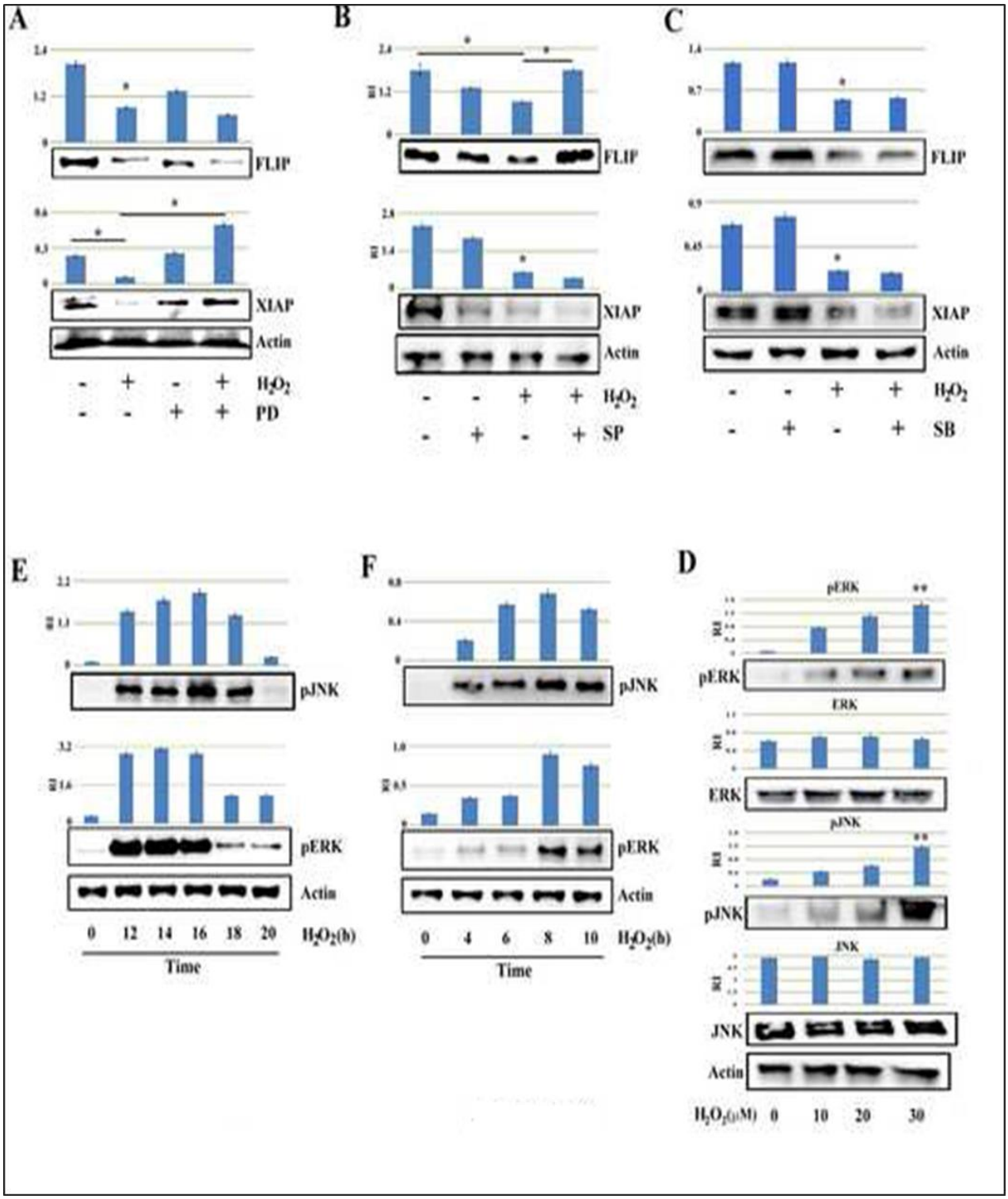

A
FLIP
XIAP
Actin
- + - + H_2O_2
- - + + PD
B
FLIP
XIAP
Actin
- - + + H_2O_2
- + - + SP
C
FLIP
XIAP
Actin
- - + + H_2O_2
- + - + SB
E
pJNK
pERK
Actin
0 12 14 16 18 20 H_2O_2(h)
Time
F
pJNK
pERK
Actin
0 4 6 8 10 H_2O_2(h)
Time
D
pERK
ERK
pJNK
JNK
Actin
H_2O_2(μM) 0 10 20 30

Figure 9

Fig 9: ERK and JNK are involved in regulation of XIAP and FLIP respectively in H_2O_2 treated K562(R) cells. (a) K562(R) cells were preincubated with 30 µM ERK inhibitor PD and then treated with H_2O_2 for 18 hrs. Whole cells lysate were subjected to western blot analysis for checking the protein level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (b) K562(R) cells were preincubated with 30 µM JNK inhibitor SP and then treated with H_2O_2 for 18 hrs. Whole cells lysate were subjected to western blot analysis for checking the protein level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.(c) K562(R) cells were preincubated with P38 inhibitor SB and then treated with H_2O_2 for 18 hrs. Whole cells lysate were subjected to western blot analysis for checking the protein level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (d,e) K562(R) cells were incubated with 30 µM H_2O_2 for indicated times. pJNK and pERK were checked by western blot analysis with the whole cell lysates. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. (f) K562(R) cells were incubated with indicated doses of H_2O_2 for 16 hours. Whole cell lysates were subjected to western blot analysis for checking the level of ERK, JNK, pERK, pJNK. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments**$p < 0.01$.

JNK, ERK played differential role in downregulation in cellular level of FLIP, XIAP:

To confirm role of ERK, JNK in XIAP, FLIP downregulation, K562(R) cells were treated with ERKsiRNA then treated with H_2O_2. Scrambled siRNA was used as negative control. Result indicated that H_2O_2 mediated XIAP downregulation partially reversed by ERKsiRNA treatment, but FLIP remains unchanged .ERK protein was decreased by ERKsiRNA (Fig10a).Similar knockdown assay was performed with JNKsiRNA. Result indicated that JNK knockdown reversed H_2O_2 mediated FLIP downregulation, XIAP remains unchanged. JNK decrease at protein level by JNKsiRNA (Fig10b). To confirm role of JNK, ERK, cells were transfected with pcDNA3FlagJNK2a1 plasmid and pcDNA3-HA-ERK2WT plasmid separately, followed treatment with H_2O_2. Result indicated that JNK overexpression down-regulated FLIP more in the pcDNA3 Flag JNK2a1 containing cells

than in empty vector containing cells. JNK overexpression did not downregulate XIAP by H_2O_2 (Fig10c). ERK overexpression decrease XIAP by ROS in pcDNA3-HA-ERK2WT cells than in empty vector containing cells. FLIP remained unchanged in both H_2O_2 treated cells irrespective of ERK overexpression (Fig10d). So, JNK and ERK differentially regulate FLIP and XIAP respectively.

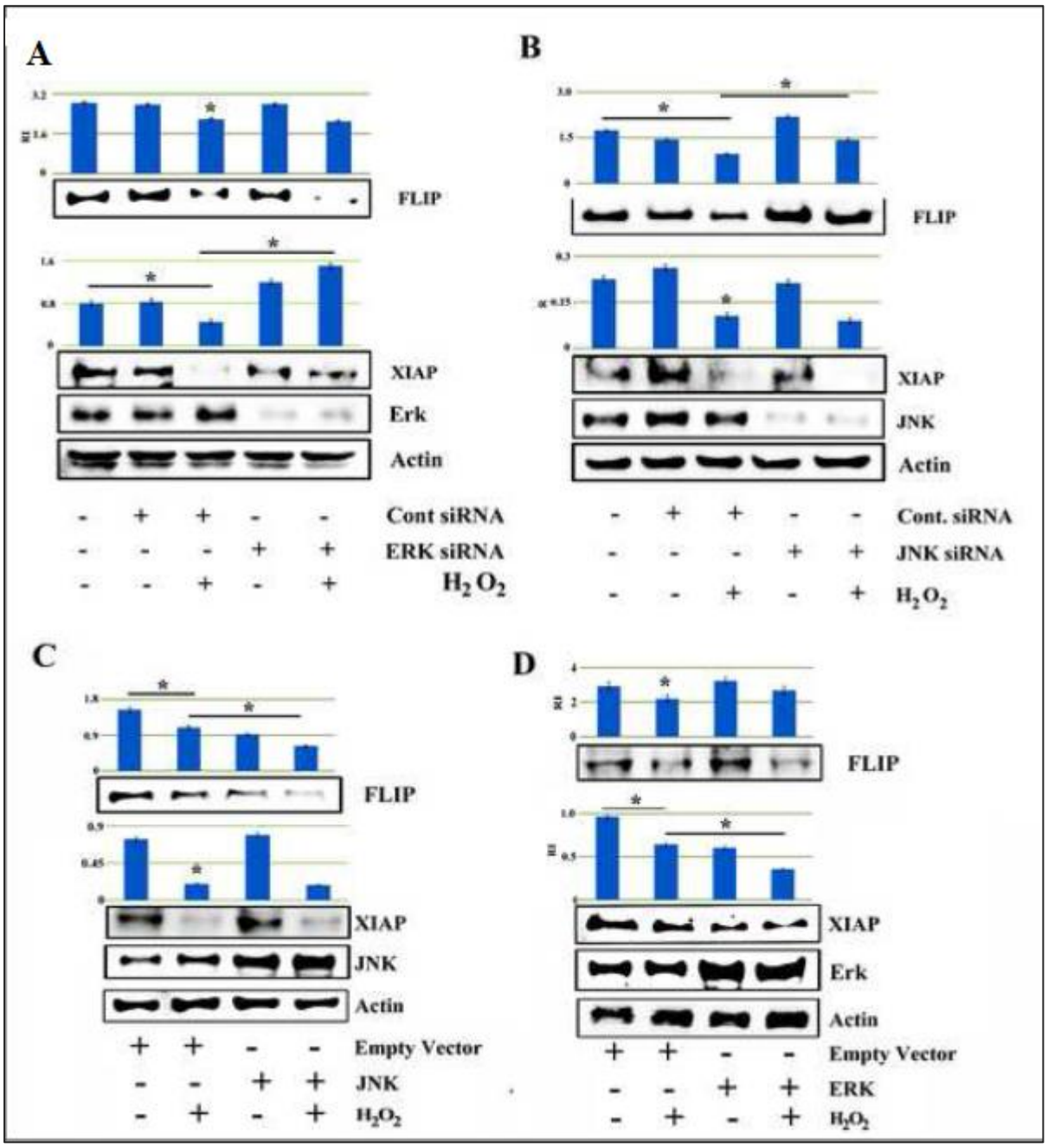


Figure 10

Fig 10: Degradation of XIAP and FLIP is ERK and XIAP mediated respectively (a) ERK was knocked down by siRNA in cells and then cells were treated with 30 μM H_2O_2.Whole cells lysate were subjected to western blot analysis for checking protein level of ERK, XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (b) JNK was knocked down by siRNA in cells and then cells were treated with 30 μM H_2O_2. .Whole cells lysate were subjected to western blot analysis for checking protein level of JNK, XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (c) K562(R) cells were transfected with either pcDNA3 Flag JNK2a1 or empty vector and then cells were treated with 30 μM H_2O_2 for 18 hrs. .Whole cells lysate were subjected to western blot analysis for checking protein level of JNK, XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (d) K562(R) cells were transfected with either pcDNA3-HA-ERK2 or empty vector and and then cells were treated with 30 μM H_2O_2 for 18 hrs. Whole cells lysate were subjected to western blot analysis for checking protein level of ERK, XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

ERK mediated PI3K and Akt dephosphorylation involved in XIAP ubiquitination

We investigated role of PI3K,Akt in XIAP downregulation. Cells were treated with PD98059, either alone or with H_2O_2. Whole cell lysate was Coimmunoprecipited with XIAP antibody, western blot was done with Akt antibody. Result indicated that amount of Akt decreased in presence of H_2O_2 alone, Akt level is reversed to level, as in control level, in presence of PD98059 (Fig11a). Result confirmed that Akt XIAP interaction is disrupted by H_2O_2, it is mediated by ERK. As PI3K, Akt were activated by phosphorylation, cells were incubated with various doses of H_2O_2, PI3K, Akt phosphorylation were decreased with increasing concentration of H_2O_2 (Fig11b). To check role of PI3K, Akt in XIAP downregulation, cells were treated with PI3K inhibitor, Wortmanin for 18 hrs. Result indicated that Wortmanin dose dependently decreased XIAP level; FLIP remained unchanged (Fig11c). Cells were pre-incubated with Wortmanin followed by H_2O_2 treatment, result showed that XIAP level decreased further than H_2O_2 alone, FLIP level similarly didn't decrease further than H_2O_2 alone (Fig11d). So, PI3K, Akt have role in XIAP downregulation, no effect on FLIP. To check role of ERK in PI3K, Akt phosphorylation, cells were treated with PD98059, then H_2O_2 treatment for 14 hrs. Result indicated that PD reversed H_2O_2-mediated dephosphorylation of PI3K and Akt (Fig11e).To confirm this data, cells were transfected with either pcDNA3-HAERK2 WT plasmid or its empty backbone, then H_2O_2 treatment, result indicated that H_2O_2 increased PI3K,Akt dephosphorylation in presence of ERK overexpression than H_2O_2 alone (Fig11f). This data confirm that H_2O_2 activated ERK, which inhibited PI3K,Akt by dephosphorylation, thus binding of Akt to XIAP was inhibited, thus degradation and ubiquitination of XIAP was occurred.

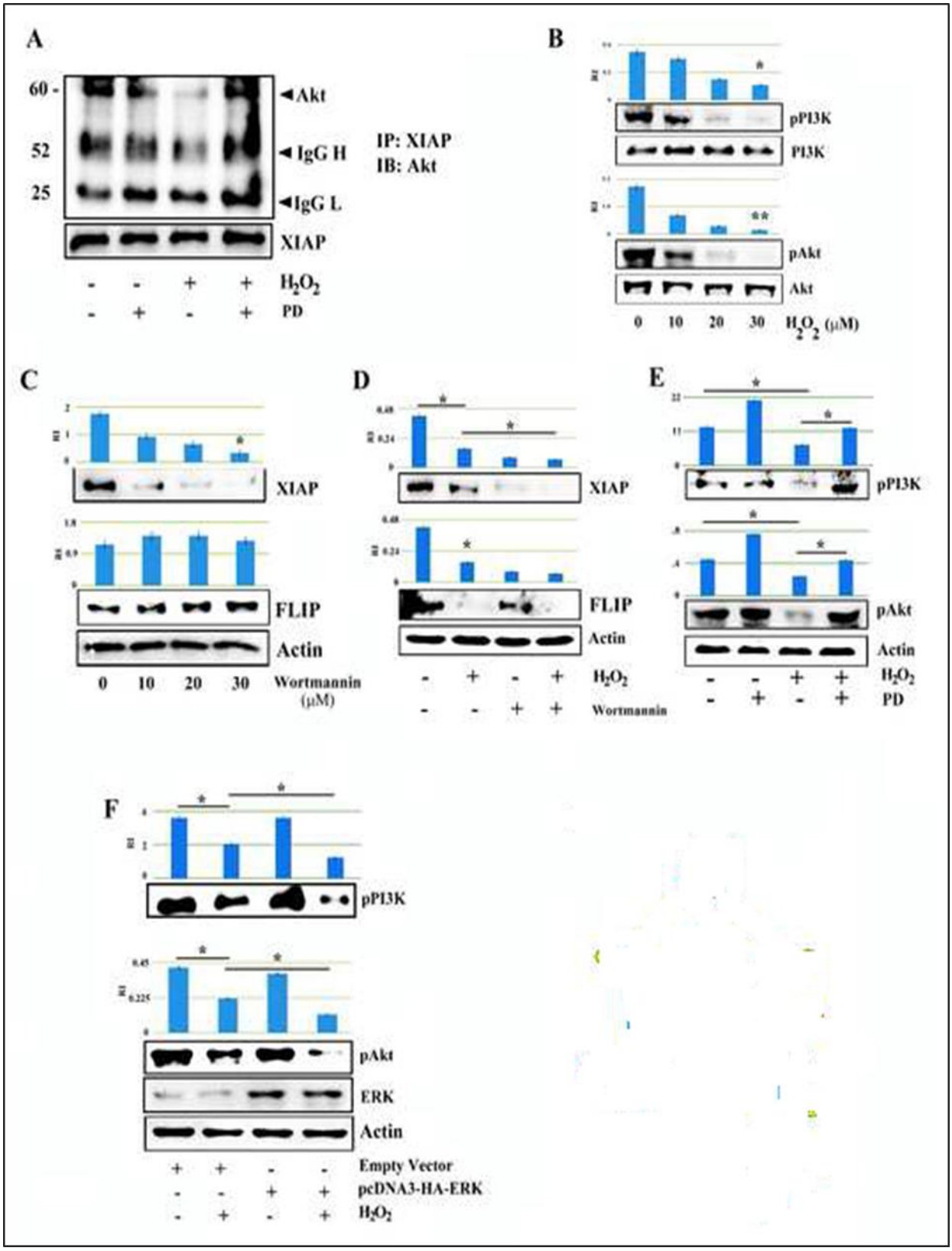

A
60 -
52
25
Akt
IgG H
IgG L
XIAP
IP: XIAP
IB: Akt
- - + + H2O2
- + - + PD
B
pPI3K
PI3K
pAkt
Akt
0 10 20 30 H2O2 (μM)
C
XIAP
FLIP
Actin
0 10 20 30 Wortmannin (μM)
D
XIAP
FLIP
Actin
- + - + H2O2
- - + + Wortmannin
E
pPI3K
pAkt
Actin
- - + + H2O2
- + - + PD
F
pPI3K
pAkt
ERK
Actin
+ + - - Empty Vector
- - + + pcDNA3-HA-ERK
- + - + H2O2

Figure 11

Fig 11: ERK mediated PI3K and Akt dephosphorylation involved in XIAP ubiquitination and degradation (a) K562 (R) cells were preincubated with PD98059 and then treated with 30μM H_2O_2 for 14 hrs. Whole cell lysates were subjected to co-immunoprecipitation with XIAP antibody and then western blot analysis were performed with anti-Akt antibody. The lower panel shows the whole cell lysates and upper panel, the immunoprecipitates.(b) K562(R) cells were treated with 30 μM H_2O_2 for 14 hours. Then whole cell lysate were subjected to western blot analysis for checking PI3K, pPI3K, Akt, pAkt. (c) K562(R) cells were treated with increasing doses of wortmannin for18 hours and then whole cell lysates were subjected to western blot analysis for checking FLIP and XIAP expression. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (d) K562(R) cells were preincubated with 10 μM wortmannin and then treated with 30 μM H_2O_2 for 18 hours. Cell lysate were subjected to western blot. XIAP and FLIP protein levels were checked.(e) K562(R) cells treated with 30 μM H_2O_2 either in presence or absence of 30 μM PD and western blot analysis was done for checking pPI3K and pAKT. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (f) K562(R) cells were transfected with either pcDNA3- HA-ERK2 or empty vector and then treated with 30μM H_2O_2 for 18 hrs. Western blot analysis was done for checking pPI3K, pAkt and ERK level. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

ITCH is responsible for JNK mediated FLIP ubiquitination and degradation:

Data indicate that H_2O_2 activated JNK, which downregulated FLIP in K562(R) cells. FLIP ubiquitination is possible by binding to ubiquitin ligase, ITCH [31,32].Cells were treated with H_2O_2 for 10hrs, cell-lysate was coimmunoprecipitated with FLIP-antibody, western blot with ITCH antibody. Result indicated that ITCH binds with FLIP in presence of H_2O_2 (Fig12a). H_2O_2 significantly increase ITCH at protein level (FIG12b). Real time PCR data reveal that ITCH expression was increased at mRNA level in presence of ROS (Fig12c). Since FLIP is downregulated by activated JNK, cells were treated with H_2O_2 alone in combination with SP600125. ITCH was coimmunoprecipitated with FLIP antibody. Coimmunoprecipitation data indicated that amount of ITCH decreased in presence of JNK inhibitor SP600125 than H_2O_2 alone (Fig12d). We checked the effect of JNK on ITCH, cells were treated with either H_2O_2 alone or in presence of SP600125. Data revealed that SP600125 reversed H_2O_2 mediated increase of ITCH (Fig12e). ERK didn't play any role in ITCH upregulation. To confirm this, cells were treated with H_2O_2 either alone or in combination with PD98059. Result showed that ITCH did not change in presence of ERK inhibitor PD than H_2O_2 alone (Fig12f). So, H_2O_2 increases ITCH at mRNA level by activation of JNK then ITCH associates with FLIP and degrades FLIP.

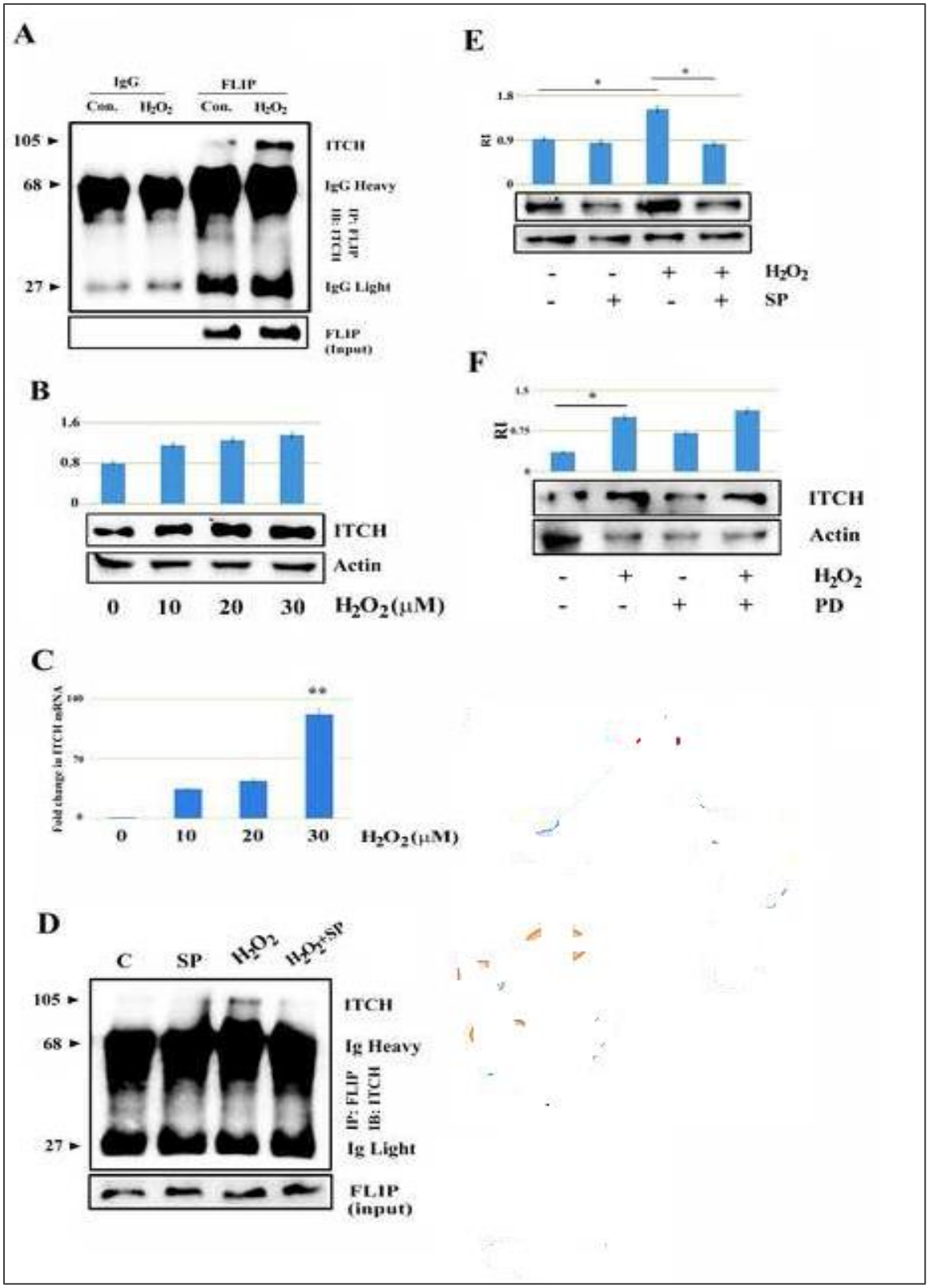

A
IgG
FLIP
Con.
H2O2
Con.
H2O2
105
68
27
ITCH
IgG Heavy
IP: FLIP
IB: ITCH
IgG Light
FLIP
(Input)
B
1.6
0.8
0
ITCH
Actin
0
10
20
30
H2O2(μM)
C
Fold change in ITCH mRNA
100
50
0
**
0
10
20
30
H2O2(μM)
D
C
SP
H2O2
H2O2+SP
105
68
27
ITCH
Ig Heavy
IP: FLIP
IB: ITCH
Ig Light
FLIP
(input)
E
*
*
1.8
RI
0.9
0
- - + + H2O2
- + - + SP
F
*
1.5
RI
0.75
0
ITCH
Actin
- + - + H2O2
- - + + PD

Figure 12

Fig12: FLIP degradation is mediated by increased expression of ITCH (a) K562 (R) cells were treated with 30μM H_2O_2 for 11 hours and the whole cell lysates were subjected to co-Immunoprecipitation with FLIP antibody and control IgG and then western blot analysis were performed with anti-ITCH antibody. The lower panel shows the whole cell lysates and upper panel, the immunoprecipitates. (b) Cells were treated with increasing concentration of H_2O_2 for 11 hrs and whole cells lysate were subjected with western blot analysis for checking ITCH at protein level. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (c) K562(R) cells were treated with increasing doses of H_2O_2 and real time PCR was performed for checking ITCH expression at protein level. Data was normalized with actin as housekeeping gene and presented graphically as fold change relative to control. Data indicates mean ± SD of three independent experiments. **$p < 0.01$ (d) K562 (R) cells were treated with either 30μM H_2O_2 alone or in combination of SP600125 for 11 hours and the whole cell lysates were subjected to co-Immunoprecipitation with FLIP antibody and then western blot analysis were performed with anti-ITCH antibody. The lower panel shows the whole cell lysates and upper panel, the immunoprecipitates.(e) K562 (R) cells were treated with SP600125 alone or in combination of H_2O_2. Western blot analysis was done for checking the level of ITCH at protein level. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (f) K562 (R) cells were treated with PD98059 alone or in combination of H_2O_2. Western blot analysis was done for checking the level of ITCH at protein level. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

From objective II, it is confirmed that ROS downregulates XIAP, FLIP by ubiquitin proteosomal pathway. ROS-activated ERK decreases Akt phosphorylation that inhibits binding of Akt to XIAP. ROS increases ITCH at protein level by activation of JNK. ITCH (E3 ubiquitin ligase) associates with FLIP and degrades it by ubiquitin-proteasome pathway.

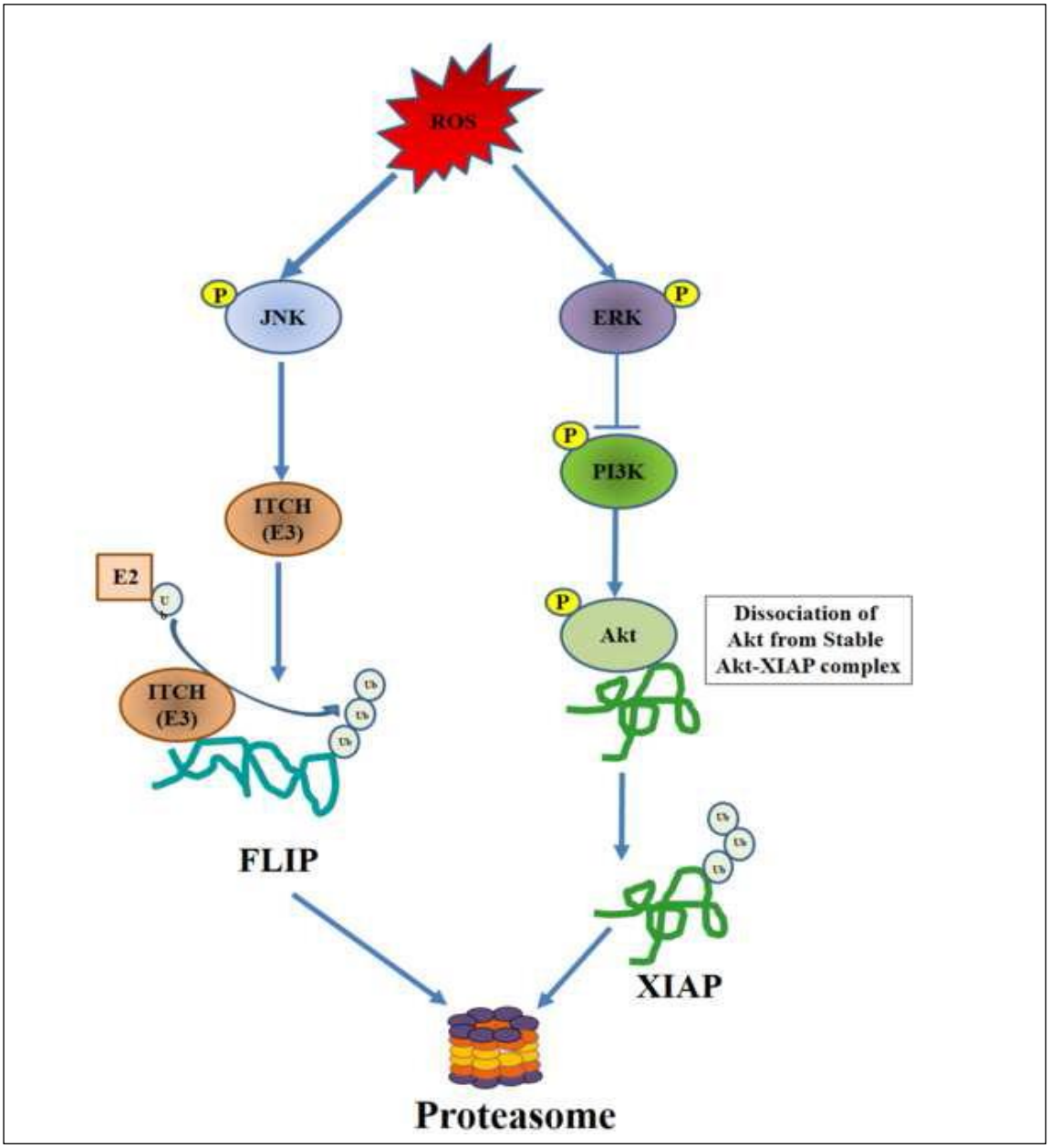


Figure 13

Figure 13 : ROS mediated XIAP,FLIP proteosomal degradation via MAPK kinase pathway in imatinib resistant K562 cells. ROS decreases anti-apoptotic protein XIAP and FLIP by activation of ubiquitin proteasomal pathway. ROS-activated ERK subsequently decreases Akt phosphorylation which inhibits the binding of Akt to the XIAP and increases its ubiquitin mediated degradation. On the other hand ROS increases ITCH at protein level by activation of JNK. ITCH, being an E3 ubiquitin ligase, associates with FLIP and degrades it by ubiquitin-proteasome pathway

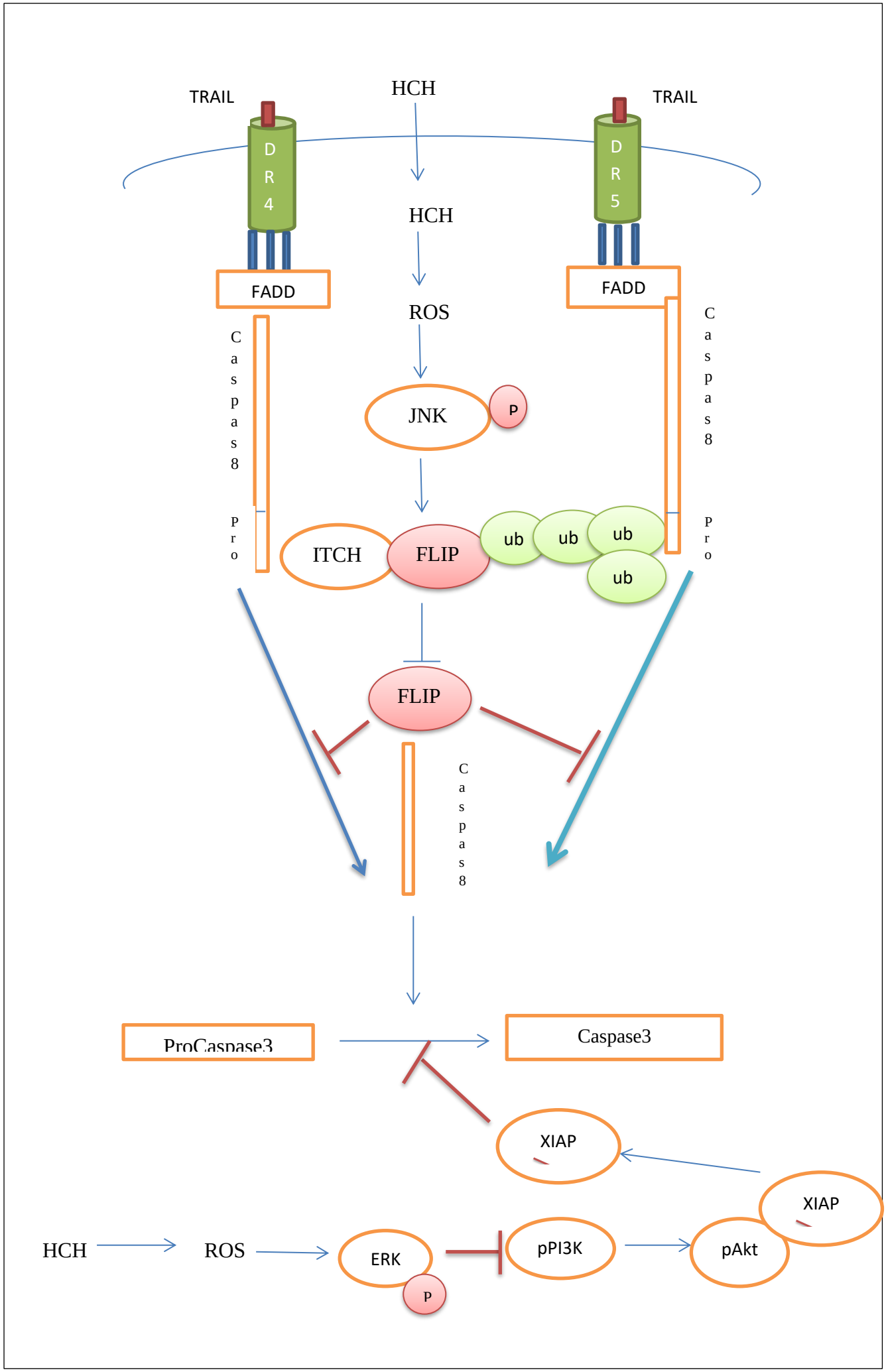

TRAIL
HCH
TRAIL
DR4
DR5
HCH
FADD
FADD
ROS
Caspas8
Pro
JNK
P
Caspas8
Pro
ITCH
FLIP
ub
ub
ub
ub
FLIP
Caspas8
ProCaspase3
Caspase3
XIAP
XIAP
HCH
ROS
ERK
P
pPI3K
pAkt

Fig: Hydroxychavicol downregulates anti apoptotic protein XIAP,FLIP via ROS generation. MAPK pathway involved in this downregulation in imatinib resistant K562 cells. HCH induces ROS inside cells specially H_2O_2. . ROS decreases anti-apoptotic protein XIAP and FLIP by activation of ubiquitin proteasomal pathway. ROS-activated ERK subsequently decreases Akt phosphorylation which inhibits the binding of Akt to the XIAP and increases its ubiquitin mediated degradation. On the other hand ROS increases ITCH at protein level by activation of JNK. ITCH, being an E3 ubiquitin ligase, associates with FLIP and degrades it by ubiquitin-proteasome pathway. In this way, ROS downregulates XIAP and FLIP . HCH sensitize imatinib-resistant CML cells to TRAIL-induced apoptosis through downregulation of XIAP and FLIP via ROS generation.

Objective III: **To check the key findings in imatinib resistant cells from objective I and objective II in imatinib sensitive cells.**

The followings are the key findings from object I and II.

1. Hydroxychavicol downregulates XIAP, FLIP via ROS generation.
2. H_2O_2 downregulates XIAP, FLIP by ubiquitin proteosomal pathway.
3. H_2O_2 mediated ERK and JNK activation cause XIAP and FLIP downregulation respectively.
4. ROS activated ERK decreases PI3K/Akt phosphorylation that inhibit binding of Akt to XIAP. So, XIAP autoubiquitination is possible.
5. ROS increases ITCH expression at protein level by activation of JNK. ITCH associates with FLIP and degrades it by ubiquitin proteosome pathway.

Next, the above key results from imatinib resistant CML cell line K562 have now been checked in Imatinib-sensitive CML cell lines K562.

H_2O_2 downregulates XIAP, FLIP in Imatinib sensitive cells by lysosomal degradation pathway

To check the effect of Hydroxychavicol on XIAP, FLIP downregulation in imatinib sensitive cells, cells are treated with different doses of Hydroxychavicol. Result show that XIAP, FLIP downregulation were increased with increasing concentration of Hydroxychavicol (Fig14a). Hydroxychavicol downregulates XIAP, FLIP in ROS dependant manner, specifically H_2O_2 dependant manner. To check effect of ROS on XIAP, FLIP downregulation, both cells lines were treated with 30 μM H_2O_2. Result indicates that H_2O_2 decreases XIAP and FLIP at protein level (Fig14b). We observed that XIAP and FLIP both level are decreased in imatinib resistant cells. When cells were pretreated with NAC followed by H_2O_2, NAC significantly reversed XIAP, FLIP downregulation (Fig14c). To understand the molecular mechanism behind FLIP, XIAP downregulation, K562(S) were treated with MG132 before incubation with H_2O_2, result showed that MG132 didn't reverse H_2O_2 mediated XIAP and FLIP downregulation (Fig14d). So, H_2O_2 mediated downregulation of FLIP, XIAP is not proteosomal mediated in k562 (S) cells. In contrast, H_2O_2 mediated XIAP, FLIP downregulation is occurred via proteosomal dependant in K562 (R) cells.

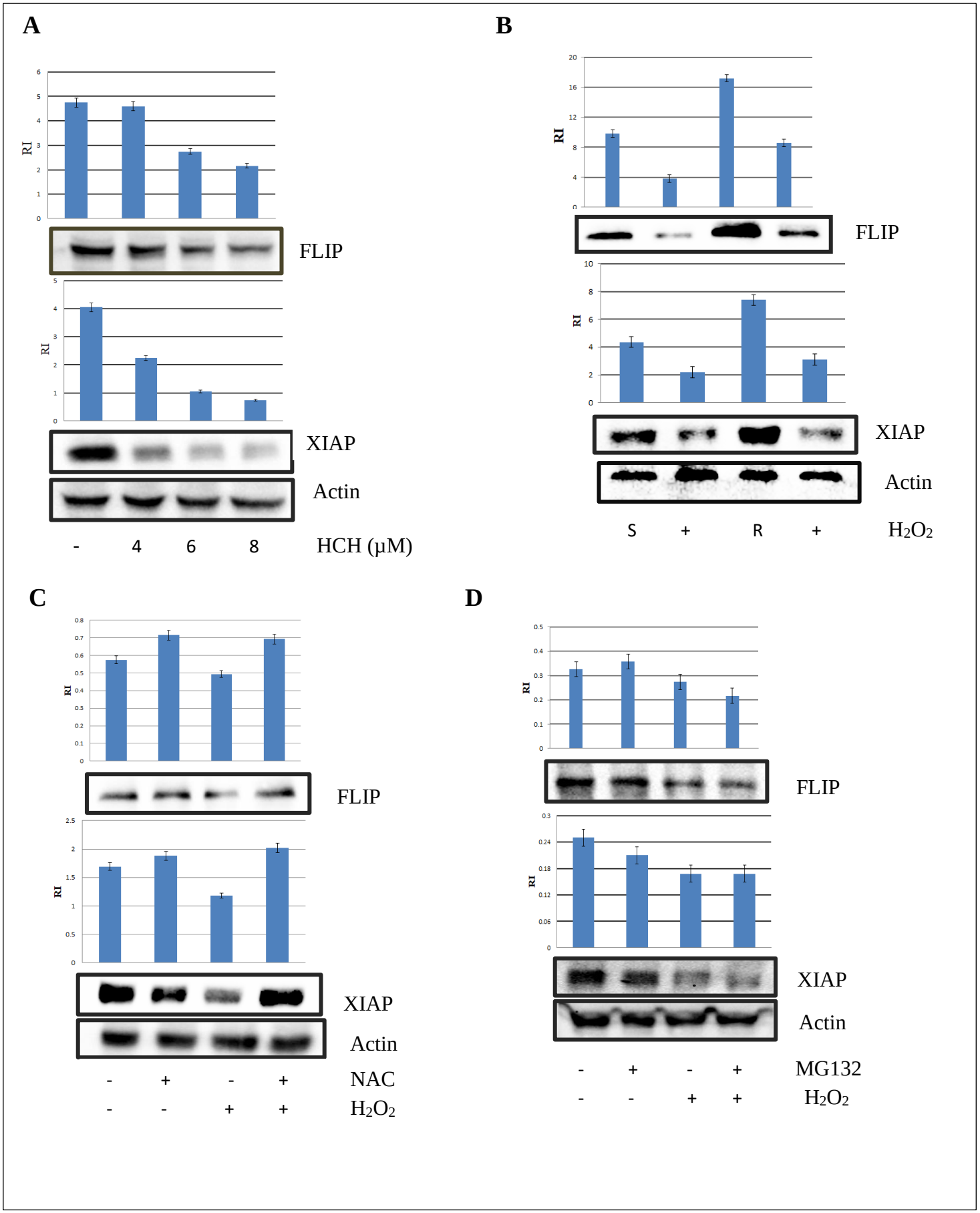

A
RI
FLIP
XIAP
Actin
- 4 6 8
HCH (μM)
B
RI
FLIP
XIAP
Actin
S + R +
H2O2
C
RI
FLIP
XIAP
Actin
- + - +
NAC
- - + +
H2O2
D
RI
FLIP
XIAP
Actin
- + - +
MG132
- - + +
H2O2

Figure 14

Fig14: H_2O_2 downregulates XIAP, FLIP in Imatinib sensitive cells via lysosomal degradation pathway. a) K562(S) cells were treated with different doses of Hydroxychavicol for 18 hrs. Western blot analysis was done for checking the level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis. b) Imatinib sensitive K562 cells and imatinib resistant K562 cells were treated with H_2O_2 for 18 hrs. Western blot analysis was done for checking the level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis**.** C) K562 cells were pre-incubated with 1mM NAC for 1 hr and then treated with 30μM H_2O_2 for overnight. Whole cells lysate were subjected to western blot analysis for checking protein level of XIAP, FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.d) Imatinib sensitive K562 cells were treated with MG132 followed by treated with H_2O_2 for 18 hrs. Western blot analysis was done for checking the level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

ROS is involved in activation of JNK but downregulation of ERK activity in imatinib sensitive cells:

We have checked whether ROS can modulate MAPK pathway. So, K562 (S) cells were treated with different doses of H_2O_2. Result showed that H_2O_2 increase JNK phosphorylation but decrease ERK phosphorylation in K562(S) cells. (Fig 15a).In contrast, H_2O_2 increases phosphorylation of JNK and ERK in K562(R).

A

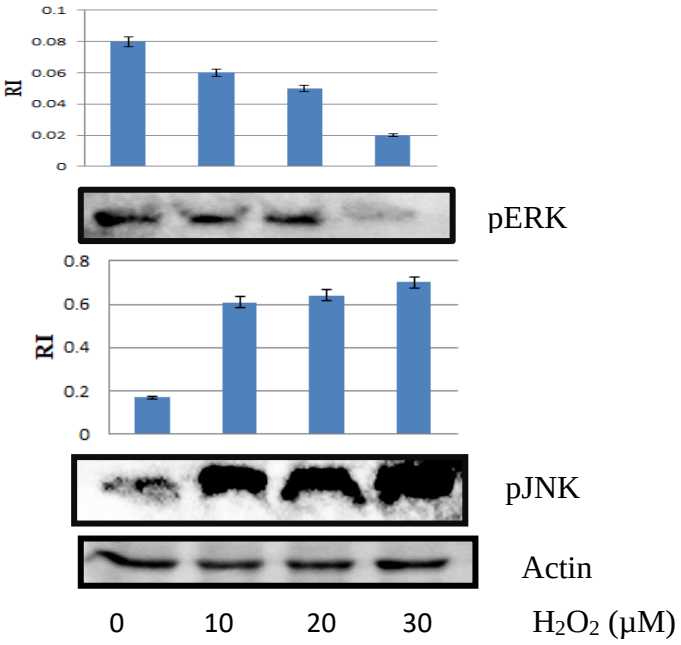


Figure 15

Fig15 : ROS is involved in activation of JNK but downregulation of ERK activity a) Imatinib sensitive K562 cells were treated with H_2O_2 for for 14 hrs. Western blot analysis was done for checking the level of phosphorylation level of JNK and ERK. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

Activated JNK involved in XIAP and FLIP downregulation both in K562(S) cells:

We checked whether JNK pathway was involved in this downregulation. Cells were incubated with SP600125 then treated with H_2O_2. Result indicated that SP reversed H_2O_2 mediated FLIP, XIAP down-regulation after 18 hrs (Fig16a). So, ROS mediated activated JNK is involves in XIAP and FLIP downregulation.

A

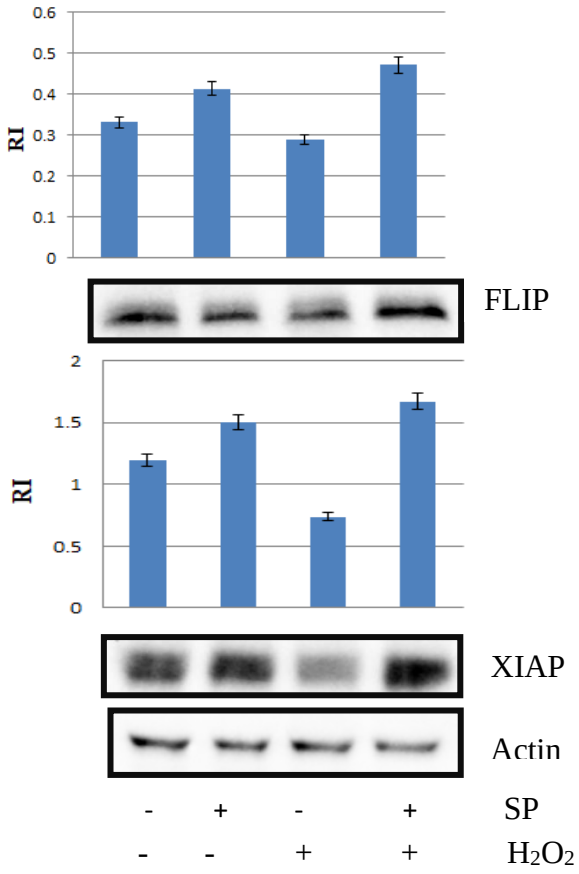


Figure 16

Fig16: Activated JNK is involved in XIAP and FLIP downregulation both in Imatinib-sensitive K562 cells a) Imatinib sensitive K562 cells were treated with SP followed by treated with H_2O_2 for 18 hrs. Western blot analysis was done for checking the level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

Activated ERK involved in XIAP and FLIP upregulation in K562 (S) cells:

Cells were incubated with PD98059, then treated with H_2O_2 for 18 hrs. Result indicated that PD increases H_2O_2 mediated FLIP, XIAP downregulation (fig 17a). It is concluded that in absence of ROS, ERK upregulates XIAP and FLIP.

A

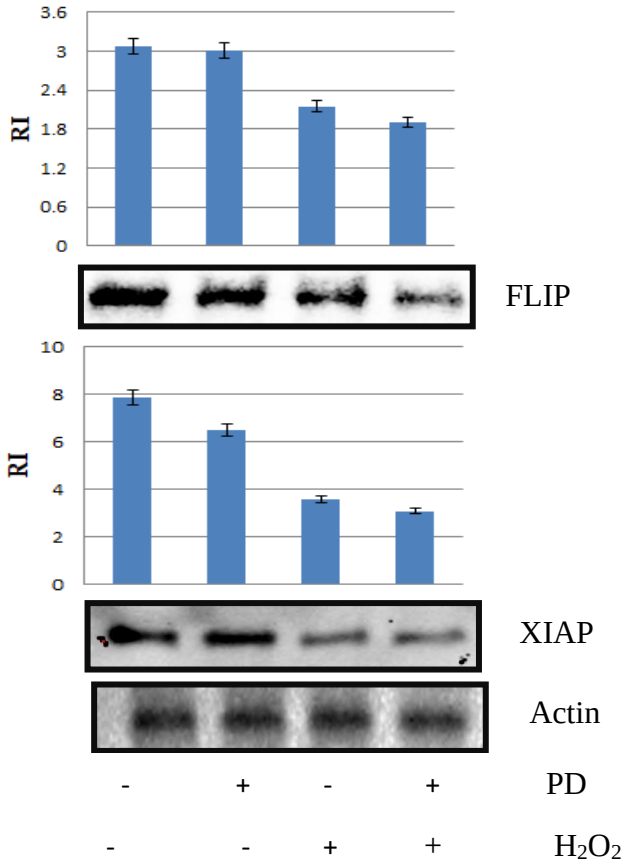


Figure 17

Fig17: Activated ERK involved in XIAP and FLIP upregulation in Imatinib-sensitive K562 cells: **a)** Imatinib sensitive K562 cells were treated with PD followed by treated with H_2O_2 for 18 hrs. Western blot analysis was done for checking the level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

H_2O_2 do not increase ITCH expression in K562 (S) cells:

To check the effect of ROS on ITCH, K562(S) cells were treated with different doses of H_2O_2 for 10 hours and result showed that ITCH is little bit decreased at protein level at 30 µM H_2O_2 (Fig18a). So, ITCH has no role in XIAP, FLIP degradation in K562 (S) cells. In-contrast, H_2O_2 increase expression of ITCH in K562(R) cell, play role in FLIP degradation.

A

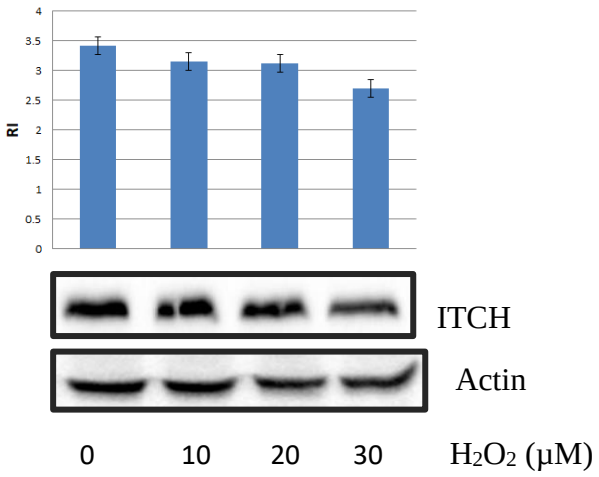


Figure 18

Fig18**:** H_2O_2 do not increase ITCH expression in Imatinib sensitive K562 cells: a) Immunobloting of ITCH was performed using whole cell lysate of K562 (S) cells under indicated concentration of H_2O_2 treatment for 11 hours. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

H_2O_2 mediated ERK dephosphorylation causes PI3K, Akt dephosphorylation, thereby enhances XIAP, FLIP downregulation in K562 (S) cell

Cells were treated with different doses of H_2O_2 for 14 hrs. Data indicated that H_2O_2 dose dependently decreased both PI3K and Akt phosphorylation level whereas Akt remains unaltered at protein level (Fig19a). To check the role of PI3K and Akt in XIAP, FLIP downregulation, cells were treated with PI3K inhibitor Wortmannin and then H_2O_2 was added, Result showed that XIAP, FLIP level decreased further than H_2O_2 alone (Fig19b). So, it is confirmed that PI3K and Akt played a crucial role in XIAP,FLIP upregulation in K562(S) cells. In contrast, PI3K, Akt played role in XIAP upregulation with minimal effect on FLIP in K562(R) cells. To check role of PI3K, Akt in XIAP downregulation, cells were treated with Wortmanin for 18 hrs. Result indicated that Wortmanin dose dependently

decreased XIAP and FLIP level (Fig19c). To assess the role of ERK in PI3K and Akt phosphorylation, K562(S) cells were treated with PD98059 then treated with H_2O_2 for 14 hours. Result indicated that PD enhances H_2O_2-mediated dephosphorylation of PI3K and Akt (Fig19d). So, In H_2O_2 treated K562(S) cells, H_2O_2 inactivate ERK, thereby Pi3k, Akt is not phosphorylated, So, XIAP,FLIP degradation is occurred.

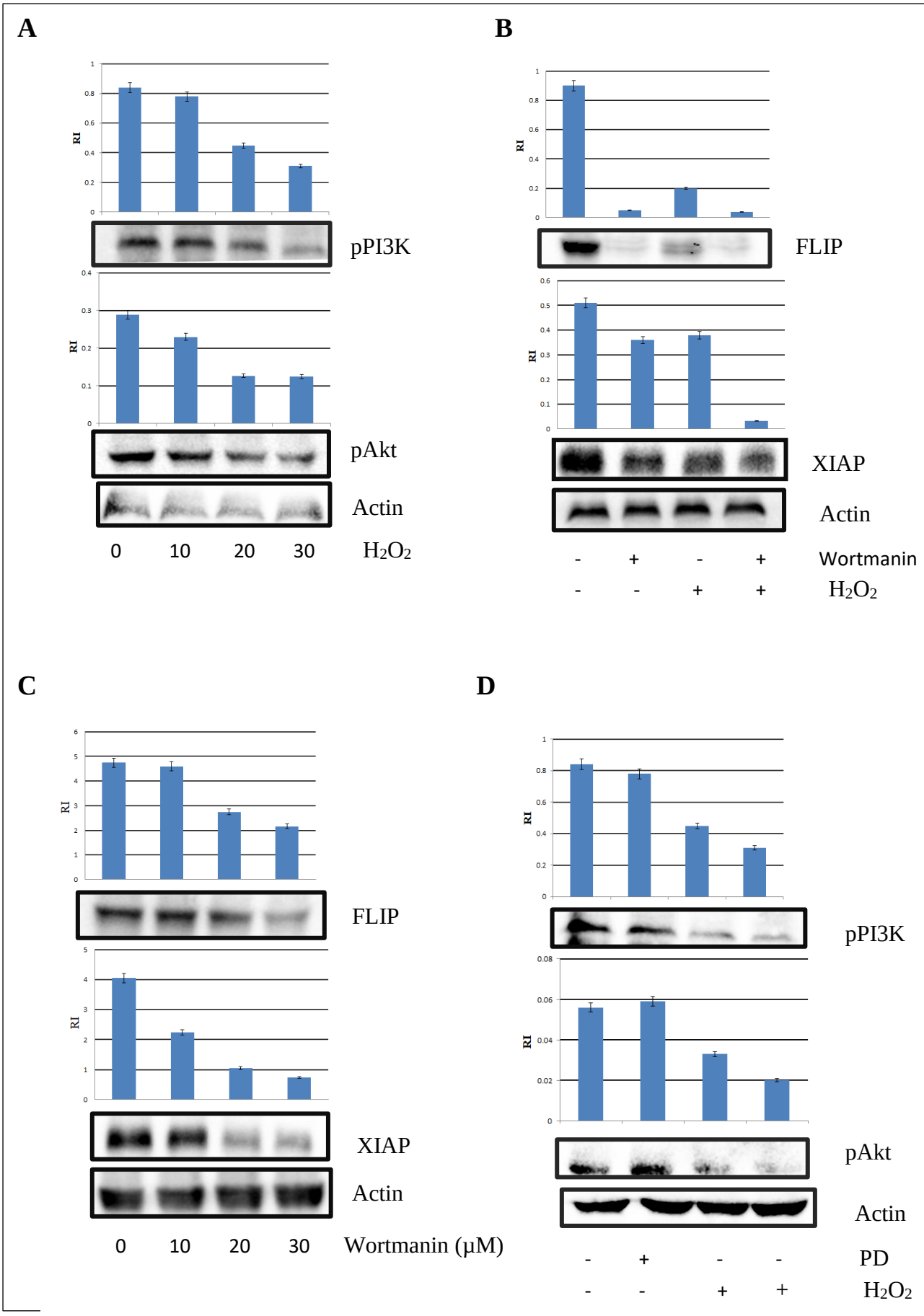

A
RI
pPI3K
pAkt
Actin
0 10 20 30 H2O2
B
RI
FLIP
XIAP
Actin
- + - + Wortmanin
- - + + H2O2
C
RI
FLIP
XIAP
Actin
0 10 20 30 Wortmanin (μM)
D
RI
pPI3K
pAkt
Actin
- + - - PD
- - + + H2O2

Figure 19

Fig19: H_2O_2 mediated ERK dephosphorylation causes PI3K, Akt dephosphorylation, thereby enhance XIAP, FLIP downregulation in imatinib sensitive K562 cell a) K562(S) cells were treated with different doses of H_2O_2 for 14 hours and then whole cell lysates were subjected to immune-blot analysis with pPI3K, pAkt antibodies. Data were presented graphically as ratio to actin after densitometry analysis. b) K562(S) cells were incubated with or without 10 μM wortmannin and 30 μM H_2O_2 for 18 hours, then cell lysates were subjected to western blot and XIAP and FLIP protein levels were checked. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis. c) K562(S) cells were treated with increasing doses of Wortmannin for18 hours and then whole cell lysates were subjected to western blot analysis for checking FLIP and XIAP expression. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis. D) K562(R) cells treated with 30 μM H_2O_2 either in presence or absence of 30 μM PD and the levels of pPI3K and pAKT was analysed by Immunoblot. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

Our data suggests that ROS decreases XIAP and FLIP in K562(S) cells. In contrast, ROS degrades XIAP and FLIP by proteosomal degradation pathway in K562(R) cells. ROS inactivates ERK subsequently decreases Akt phosphorylation thereby degradation of FLIP, XIAP are occurred in K562(S) cells. In contrast, ERK mediated dephosphorylation of PI3K and Akt plays a critical role in XIAP ubiquitination and degradation in presence of H2O2 in K562(R) cells. ROS activates JNK by phosphorylation thereby degradation of XIAP, FLIP is possible. ITCH is not involved in ROS mediated XIAP.FLIP downregulation. Because ITCH expression is not increased in presence of ROS in K562(S) cells. In contrast, in K562 (R), ROS increases ITCH at protein level by activation of JNK. ITCH associates with FLIP

and degrades it. In this way ROS downregulates FLIP and XIAP in K562(S) and K562(R) cells.

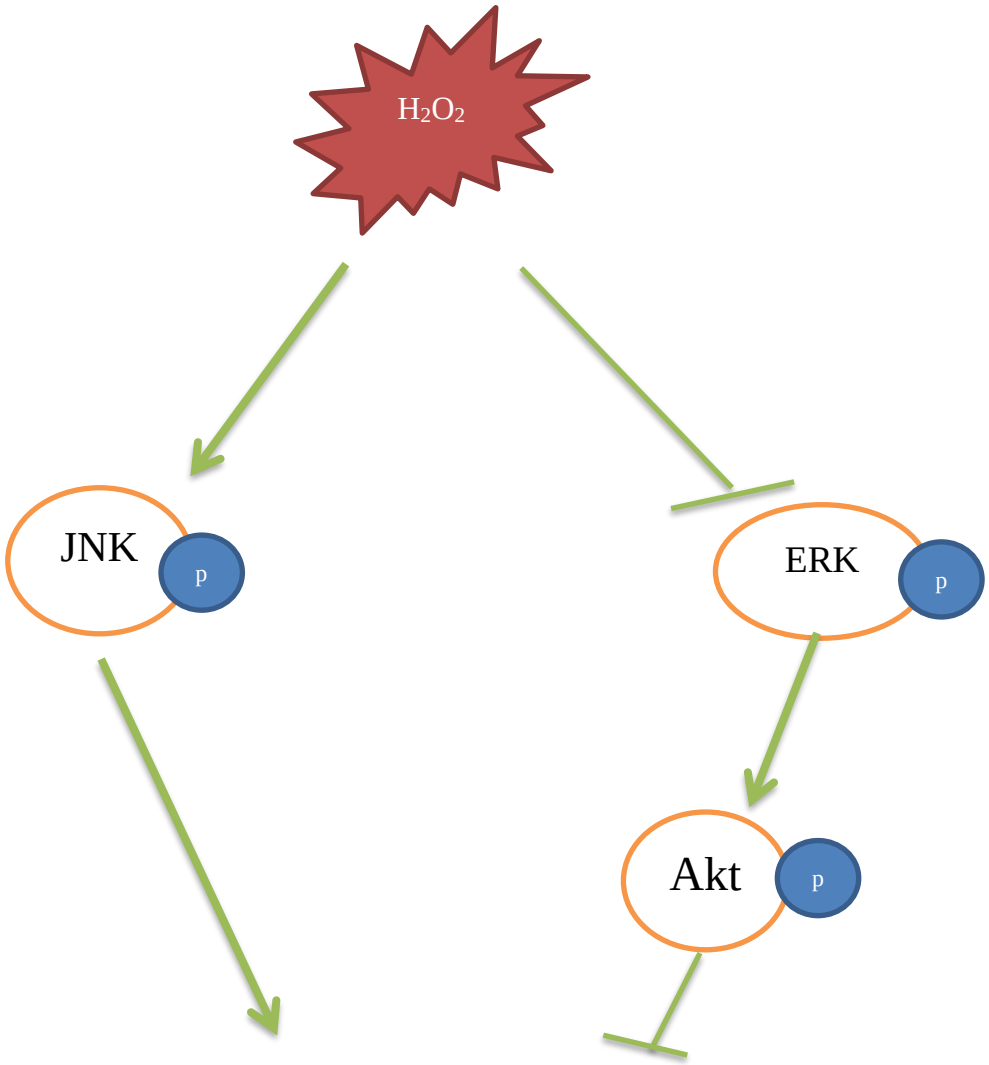


Fig: ROS mediated XIAP , FLIP degradation via MAPK kinase pathway in imatinib sensitive K562 cells

Discussion: K562 (R) cells show resistant to TRAIL mediated apoptosis. Since in a previous study Hydroxychavicol has been shown to kill K562(S) cells, we tried to sensitize K562(R) to TRAIL by using Hydroxychavicol. Combinatory treatment of HCH ,TRAIL induced K562(R) cells to TRAIL mediated apoptosis. HCH decreases XIAP, FLIP at protein level.

The down-regulation of XIAP and FLIP are responsible for TRAIL-mediated apoptosis because knocking down XIAP and FLIP by siRNA also similarly increased TRAIL-induced apoptosis. In previous study, HCH showed to generate ROS (33). Here in this study, its was found that ROS was induced by HCH. This ROS played role in the enhancement of apoptosis by TRAIL. Exogenous ROS that was applied to K562(R) cells also decreased XIAP and FLIP levels which confirms ROS as a player in the downregulation of XIAP and FLIP. So, from objective I, it is confirmed that IAPs can targeted by HCH via H_2O_2 dependant manner to sensitize K562(R) cells to TRAIL mediates apoptosis.

In objective II, we examined the signalling mechanism that is involved in H_2O_2 dependant XIAP, FLIP downregulation. ROS decreases FLIP, XIAP by activation of ubiquitin proteasomal pathway. ROS-activated ERK subsequently decreases Akt phosphorylation which inhibits binding of Akt to the XIAP, increases its ubiquitin mediated degradation. ROS increases ITCH at protein level by activation of JNK. ITCH, an E3 ubiquitin ligase, associates with FLIP cause degradation of FLIP by ubiquitin-proteasome pathway. So ROS degrades XIAP,FLIP.

We have found some key finding in K562(S) cells from objective I ,II in K562(R) cells . ROS degrades XIAP,FLIP in imatinib sensitive K562 cells as similar to K562 (R). But ROS mediated XIAP,FLIP degradation pathways are different between K562(S) and K562(R) cells. In K562(S) cells, ROS inactivates ERK pathway, thereby Akt dephosphorylation is occurred, XIAP,FLIP degradation is possible. Activated ERK is involved in XIAP,FLIP upregulation. But in K562(R) cells, ROS activated ERK involved XIAP degradation. In K562(S) cells, ROS activated JNK is involved in XIAP, FLIP degradation. In K562(R) cells, activated JNK is involved in degradation of FLIP by ubiquitination proteosomal mediated pathway.

Therapeutic implications of this study:

XIAP and FLIP both are antiapoptotic proteins. So, downregulation of these two proteins lead to apoptosis in K562 cells. In our study it has been shown that downregulation of these two proteins leads to TRAIL induced apoptosis in imatinib resistant K562 cells. In our study we also have targeted PI3K/Akt for anti leukemic treatment in imatinib resistant K562 cells. This might lead to the alternative approach for the treatment of Imatinib resistant leukemic patients who are otherwise also resistant to TRAIL.

Future Direction: We studied that ROS regulates MAPK kinase signaling pathway in Imatinib sensitive and resistant K562 cells. We studied that the mechanisms by which ROS mediated MAPK kinase signaling pathway regulate antiapoptotic proteins FLIP, XIAP expression. In future, we will find out several signaling pathways other than MAPK pathway that were regulated by ROS. Then we will study the mechanisms by which these ROS mediated signalling pathways regulates several proapoptotic proteins and antiapoptotic proteins.

Reference

1. Savona M, Talpaz M. Getting to the stem of chronic myeloid leukaemia. Nat Rev Cancer 2008; 8:341–350.

2. Aggarwal BB. Signalling pathways of the TNF superfamily: a double edged sword. Nat Rev Immunol 2003; 3:745–756.

3. S. Fulda, A.M. Gorman, O. Hori, A. Samali, Cellular stress responses: Cell survival and cell death, Int. J. Cell Biol. (2010)

4. J.M. Vicencio, L. Galluzzi, N. Tajeddine, C. Ortiz, A. Criollo, E. Tasdemir, E. Morselli, A. Ben Younes, M.C. Maiuri, S. Lavandero, G. Kroemer, Senescence, apoptosis or autophagy? When a damaged cell must decide its path - A mini-review, Gerontology. 54 (2008) 92–99.

5. K. Labbé, M. Saleh, Cell death in the host response to infection, Cell Death Differ. 15 (2008) 1339–1349.

6. C. Chen, T.S. Liu, S.C. Zhao, W.Z. Yang, Z.P. Chen, Y. Yan, XIAP impairs mitochondrial function during apoptosis by regulating the Bcl-2 family in renal cell carcinoma., Exp. Ther. Med. 15 (2018) 4587–4593.

7. L. Flanagan, J. Kehoe, J. Fay, O. Bacon, A.U. Lindner, E.W. Kay, J. Deasy, D.A. McNamara, J.H.M. Prehn, High levels of X-linked Inhibitor-of-Apoptosis Protein (XIAP) are indicative of radio chemotherapy resistance in rectal cancer., Radiat. Oncol. 10 (2015) 131.

8. L. Humphreys, M. Espona-Fiedler, D.B. Longley, FLIP as a therapeutic target in cancer., FEBS J. 285 (2018) 4104–4123.

9. S. Horn, M.A. Hughes, R. Schilling, C. Sticht, T. Tenev, M. Ploesser, P. Meier, M.R. Sprick, M. MacFarlane, M. Leverkus, Caspase-10 Negatively Regulates Caspase-8-Mediated Cell Death, Switching the Response to CD95L in Favor of NF-κB Activation and Cell Survival., Cell Rep. 19 (2017) 785–797.

10. G. Valente, F. Manfroi, C. Peracchio, G. Nicotra, R. Castino, G. Nicosia, S. Kerim, C. Isidoro, cFLIP expression correlates with tumour progression and patient outcome in non-Hodgkin lymphomas of low grade of malignancy., Br. J. Haematol. 132 (2006) 560–70.

11. M.G. Hinds, R.S. Norton, D.L. Vaux, C.L. Day, Solution structure of a baculoviral inhibitor of apoptosis (IAP) repeat, Nat. Struct. Biol. 6 (1999) 648–651

12. C. Sun, M. Cai, R.P. Meadows, N. Xu, A.H. Gunasekera, J. Herrmann, J.C. Wu, S.W. Fesik, NMR structure and mutagenesis of the third Bir domain of the inhibitor of apoptosis protein XIAP, J. Biol. Chem. 275 (2000) 33777–33781.

13. Y. Suzuki, Y. Nakabayashi, R. Takahashi, Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death., Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 8662–7.

14. Y. Yang, S. Fang, J.P. Jensen, A.M. Weissman, J.D. Ashwell, Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli, Science (80-.). 288 (2000) 874–877.

15. J. Silke, T. Kratina, D. Chu, P.G. Ekert, C.L. Day, M. Pakusch, D.C.S. Huang, D.L. Vaux, Determination of cell survival by RING-mediated regulation of inhibitor of apoptosis (IAP) protein abundance., Proc.Natl. Acad. Sci. U. S. A. 102 (2005) 16182–7.

16. H.H. Cheung, S. Plenchette, C.J. Kern, D.J. Mahoney, R.G. Korneluk, The RING domain of cIAP1 mediates the degradation of RING-bearing inhibitor of apoptosis proteins by distinct pathways, Mol. Biol. Cell. 19 (2008) 2729–2740.

17. M.A. Hughes, I.R. Powley, R. Jukes-Jones, S. Horn, M. Feoktistova, L. Fairall, J.W.R. Schwabe, M. Leverkus, K. Cain, M. MacFarlane, Co-operative and Hierarchical Binding of c-FLIP and Caspase-8: A Unified Model Defines How c-FLIP Isoforms Differentially Control Cell Fate.,

18. K. Schleich, J.H. Buchbinder, S. Pietkiewicz, T. Kähne, U. Warnken, S. Öztürk, M. Schnölzer, M. Naumann, P.H. Krammer, I.N. Lavrik, Molecular architecture of the

DED chains at the DISC: regulation of procaspase-8 activation by short DED proteins c-FLIP and procaspase-8 prodomain., Cell Death Differ. 23 (2016) 681–94.

19. C. Haag, D. Stadel, S. Zhou, M.G. Bachem, P. Möller, K.M. Debatin, S. Fulda, Identification of c-FLIPL and c-FLIPS as critical regulators of death receptor-induced apoptosis in pancreatic cancer cells, Gut. 60 (2011) 225–237.

20. T.W. Day, S. Huang, A.R. Safa, c-FLIP knockdown induces ligand-independent DR5-, FADD-, caspase-8-, and caspase-9-dependent apoptosis in breast cancer cells., Biochem. Pharmacol. 76 (2008) 1694–704.

21. X. Zhang, L. Zhang, H. Yang, X. Huang, H. Otu, T.A. Libermann, W.C. DeWolf, R. Khosravi-Far, A.F. Olumi, c-Fos as a proapoptotic agent in TRAIL-induced apoptosis in prostate cancer cells, Cancer. 67(2007) 9425–9434

22. Aggarwal BB. Signalling pathways of the TNF superfamily: a doubleedged sword. Nat Rev Immunol 2003; 3:745–756.

23. Aggarwal BB, Bhardwaj U, Takada Y. Regulation of TRAIL-induced apoptosis by ectopic expression of antiapoptotic factors. Vitam Horm 2004; 67:453–483.

24. Biswas N, Mahato SK, Chowdhury AA, Chaudhuri J, Manna A, Vinayagam J, et al. ICB3E induces iNOS expression by ROS-dependent JNK and ERK activation for apoptosis of leukemic cells. Apoptosis 2012; 17:612–626.

25. Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: cell survival and cell death. Int J Cell Biol 2010; 2010:214074.

26. Sakamoto S, Kyprianou N. Targeting anoikis resistance in prostate cancer metastasis. Mol Aspects Med 2010; 31:205–214.

27. Silke J, Meier P. Inhibitor of apoptosis (IAP) proteins-modulators of cell death and inflammation. Cold Spring Harb Perspect Biol 2013; 5:a008730.

28. Lopes RB, Gangeswaran R, McNeish IA, Wang Y, Lemoine NR. Expression of the IAP protein family is dysregulated in pancreatic cancer cells and is important for resistance to chemotherapy. Int J Cancer 2007; 120:2344–2352.

29. Hinz S, Trauzold A, Boenicke L, Sandberg C, Beckmann S, Bayer E, et al. Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAILreceptor mediated apoptosis. Oncogene 2000; 19:5477–5486

30. Fulda S, Küfer MU, Meyer E, van Valen F, Dockhorn-Dworniczak B, Debatin KM. Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer. Oncogene 2001; 20:5865–5877.

31. Eggert A, Grotzer MA, Zuzak TJ, Wiewrodt BR, Ho R, Ikegaki N, Brodeur GM. Resistance to tumornecrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with aloss of caspase-8 expression. Cancer Res 2001; 61:1314–1319.

32. Hao XS, Hao JH, Liu FT, Newland AC, Jia L. Potential mechanisms of leukemia cell resistance to TRAIL-induced apopotosis. Apoptosis 2003; 8:601–607.

33. Ashkenazi A, Dixit VM. Apoptosis control by death and decoy receptors. Curr Opin Cell Biol 1999; 11:255–260.

34. MacFarlane M. TRAIL-induced signalling and apoptosis. Toxicol Lett 2003; 139:89–97.

35. Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: cell survival and cell death. Int J Cell Biol 2010; 2010:214074.

36. Sakamoto S, Kyprianou N. Targeting anoikis resistance in prostate cancer metastasis. Mol Aspects Med 2010; 31:205–214.

37. Geiger TR, Peeper DS. Metastasis mechanisms. Biochim Biophys Act 2009; 1796:293–308.

38. Fulda S. Tumor resistance to apoptosis. Int J Cancer 2009; 124:511–515

39. Westhoff MA, Fulda S. Adhesion-mediated apoptosis resistance in cancer. Drug Resist Updat 2009; 12:127–136

Plagiarism Certificate

Curiginal

Document Information

Analyzed document	5000 words only.docx (D121133076)
Submitted	2021-12-05T07:55:00.0000000
Submitted by	Nabendu Biswas
Submitter email	nabendu.dbs@presiuniv.ac.in
Similarity	1%
Analysis address	nabendu.dbs.presi@analysis.urkund.com

Sources included in the report

W	URL: https://www.x-mol.com/paper/1363968201831256064 Fetched: 2021-12-05T07:57:00.0000000	1
J	URL: b971d91f-e7bc-47a1-9d5a-9b1e76819851 Fetched: 2019-03-18T18:40:16.4970000	1

Publication paper

› Anticancer Drugs. 2019 Feb;30(2):167-178. doi: 10.1097/CAD.0000000000000710.

Hydroxychavicol sensitizes imatinib-resistant chronic myelogenous leukemia cells to TRAIL-induced apoptosis by ROS-mediated IAP downregulation

Tamalika Paul [1], Anupam Banerjee [1], S Vijaya Bhaskara Reddy [2], Sanjit K Mahato [3], Nabendu Biswas [1]

Affiliations + expand

PMID: 30418193 DOI: 10.1097/CAD.0000000000000710

Abstract

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of cytokine superfamily, induces apoptosis in a number of tumor cells through the activation of extrinsic apoptotic pathway but shows little or no cytotoxicity toward normal cells. However some tumor cells are inherently resistant to TRAIL-mediated apoptosis, which needs to be addressed to establish TRAIL as a potential chemotherapeutic drug. In this study, our aim was to manipulate TRAIL-apoptosis pathway by hydroxychavicol (HCH), a polyphenol from Piper betel leaf, for the induction of apoptosis in TRAIL resistant chronic myeloid leukemia cell. When imatinib-resistant K562 cells were treated with HCH, it made these K562 cells sensitive to TRAIL. It was observed that HCH downregulated antiapoptotic proteins XIAP and FLIP, whereas the expression of TRAIL receptors, DR4 and DR5, remains unchanged. Moreover, we observed that reactive oxygen species or ROS played a crucial role in the downregulation of FLIP and XIAP because ROS scavenger significantly reversed the decrease of XIAP, and FLIP. Ubiquitin-proteasome pathway was observed to play a crucial role in the downregulation of XIAP and FLIP, as proteasomal inhibitor MG132 significantly reversed the downregulation of XIAP and FLIP. In conclusion, this study demonstrates the combinatorial treatment of TRAIL and HCH as promising alternative therapeutic approach to treat the imatinib-resistant leukemia, which are also resistant to TRAIL.

› Free Radic Biol Med. 2021 Apr;166:265-276. doi: 10.1016/j.freeradbiomed.2021.02.024. Epub 2021 Feb 22.

H_2O_2 mediated FLIP and XIAP down-regulation involves increased ITCH expression and ERK-Akt crosstalk in imatinib resistant Chronic Myeloid Leukemia cell line K562

Tamalika Paul [1], Rajdeep Roy [1], Rupak Dey Sarkar [1], Samraj Sinha [1], Nabendu Biswas [2]

Affiliations + expand

PMID: 33631302 DOI: 10.1016/j.freeradbiomed.2021.02.024

Abstract

Regulation of anti-apoptotic protein FLICE-like inhibitory protein (FLIP) and X-linked inhibitor of apoptosis protein (XIAP) remains a crucial step in the cell fate determination and thus targeting these anti-apoptotic proteins could be a viable strategy for the treatment of cancer. However the regulation of FLIP and XIAP is not very well established till date. Here we have shown that ROS decreased XIAP and FLIP by activation of ubiquitin-proteasomal pathway in imatinib resistant K562 cells. Activation of the components of MAPK pathway, ERK and JNK, played a crucial role in XIAP and FLIP degradation because ectopic expression or knock down of ERK and JNK changed the pattern of ROS mediated down-regulation of these two proteins. We have also found that JNK and ERK differentially regulates FLIP and XIAP, respectively. Moreover, our data suggests that activated ERK decreased Akt phosphorylation and thus its binding to and stabilization of XIAP. On the other hand, JNK activation increased E3 ubiquitin ligase ITCH expression and its binding to FLIP which leads to its degradation. Thus, we have, for the first time elucidated that ROS mediated ERK-Akt crosstalk regulates XIAP. We have also shown for the first time that ROS regulates ITCH expression which controls FLIP degradation.

Keywords: ERK; FLIP; Itch; JNK; ROS; XIAP.

Copyright © 2021 Elsevier Inc. All rights reserved.

Conference Certificate

11th East Zonal Oncology CME

(under the banner of IASO & ASI, W.B.)

Organised by

SAROJ GUPTA CANCER CENTRE & RESEARCH INSTITUTE
THAKURPUKUR, KOLKATA

THEME : Clinical and Molecular Advances in Breast Cancer

On 22nd February | 2020

Certificate Presented to

Dr./ Mr./Ms. Tamalika Paul

"Is Certified in recognition of Poster Presentation."

Prof. (Dr.) Arnab Gupta
Chairman, Organizing Committee
President - IASO

Dr. Bitan Chattopadhyay
President
ASI (W.B.)

Prof. (Dr.) Biman Chakraborty
Chairman, Organizing Committee

Dr. Somsubhra Nath
Jt. Organizing Secretary

Dr. Syed Hassanujjaman
Jt. Organizing Secretary

"आत्मनिर्भरभारताय विज्ञानम्"
Science for Self-Reliant India and Global Welfare

INDIA INTERNATIONAL SCIENCE FESTIVAL 2020

MINISTRY OF SCIENCE AND TECHNOLOGY
MINISTRY OF EARTH SCIENCES
MINISTRY OF HEALTH & FAMILY WELFARE
GOVERNMENT OF INDIA

Certificate of Participation and Oral Presentation

This is to certify that Prof./Dr./Mrs./Mr./Ms. TAMALIKA PAUL of PRESIDENCY UNIVERSITY has participated and delivered an oral presentation entitled "HYDROXYCHAVICOL SENSITIZES IMATINIB-RESISTANT CHRONIC MYELOGENOUS LEUKEMIA CELLS TO TRAIL-INDUCED APOPTOSIS BY ROS-MEDIATED IAP DOWNREGULATION" under the theme "FRONTIERS AREA OF RESEARCH - LIFE SCIENCES" in the **Young Scientists' Conference** organized during **22-12-2020** to **24-12-2020** as a part of **India International Science Festival- 2020** by the Ministry of Science and Technology, Ministry of Earth Sciences, and Ministry of Health and Family Welfare, Govt. of India in collaboration with Vijnana Bharati (VIBHA).

Dr. Shekhar C. Mande
DG, CSIR & Secretary, DSIR

Dr. Vijay P. Bhatkar
President, Vijnana Bharati

PRESIDENCY UNIVERSITY
KOLKATA

Report on Departmental Pre-Submission Seminar for Ph.D. Programme

1	Name of the Candidate	Tamalika Paul
2	Department	Department of Life Sciences
3	Title of Thesis (As per Ph.D. Registration Certificate)	Apoptosis regulators as a target for induction of apoptosis of Imatinib resistant chronic myeloid leukemia cells
4	Date of receipt of 5000-word summary by the Convener	January 23rd, 2022
5	Date of Seminar Presentation	February 9th, 2022

Dr. Nabendu Biswas
Asst. Professor, Dept. of Life Sciences
Presidency University, Kolkata

Signature of the Supervisor

Date:

Signature of the Co-supervisor (if any)

Date:

This is to certify that (name of the candidate) presented his/her work in a Departmental Seminar in defense of his/her thesis in presence of Ph.D. Committee, Faculty members and Research/MA/MSc/BA/BSc students.

(Signature of the Convener & Members of the Departmental Ph.D. Committee)

1. Prof.Anita Mukherjee

2.Prof.Maitree Bhattacharyya

3. Prof. Prabir Kumar Mukhopadhyay

4. Prof. Devashish Sen

5. Dr. Malay Das

6. Dr. Aparna Mukhopadhyay

7. Dr. Sanghamitra Dey

8. Dr. Santanu Chakraborty

9. Dr. Puja Ray

Report on the performance of candidate: She can submit her thesis as per University rules.

Signature of the Convener of the Departmental Ph.D. Committee

Apoptosis regulators as a target for induction of apoptosis of Imatinib resistant chronic myeloid leukemia cells

Tamalika Paul

Registration Number: R-17RS18210108

Department of LIFE SCIENCE

Presidency University

Name of Supervisor: Dr. Nabendu Biswas

Designation: Assistant Professor

Name: Tamalika Paul

Supervisor Name: Dr. Nabendu Biswas

Title of Thesis: Apoptosis regulators as a target for induction of apoptosis of Imatinib resistant chronic myeloid leukemia cells

PhD registration Number: R-17RS18210108

PhD registration Date: 14TH August, 2018

Enrolment Number: PU/PHD/BIOS/2017/023

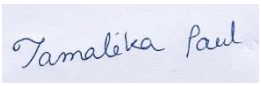
Enrolment Date: 20th September, 2017

Place of Work: Presidency University, Kolkata, West Bengal

Residential Address: 46, Rathtala colony P.O: Ranaghat, Dist: Nadia Pin code:741201

Email id:tamalika_paul@yahoo.com/tamalikapaul3@gmail.com

Phone Number: 9674086951

Signature of Research Scholar: 

Signature of Supervisor:

Contents:

Subject	**Page number**
Abstract	3
Introduction	3-4
Objectives	5
Methodology	5-7
Objective I	
Result	8-19
Objective II	
Result	20-36
Objective III	
Result	39-49
Discussion	49-50
Therapeutical implication	50
Future Direction	51
Reference	51-54
Plagiarism Certificate	
First page of published manuscript	
Certificate of conferences attended	

Apoptosis regulators as a target for induction of apoptosis of Imatinib resistant chronic myeloid leukemia cells

Abstract: Apoptosis is a regulated cell death characterized by cell shrinkage, nuclear condensation, DNA fragmentation, membrane blebbing. There are several apoptosis regulators eg: BCL2, BclXL, BAX, Survivin, FLIP, XIAP, cIAP etc. Targeting these apoptosis regulators may be a viable strategy for the treatment of cancer because one of the hallmarks of cancer is the deregulation of apoptosis. In our first study we were trying to kill chronic myelogenous Leukemia cell line K562, which were resistant to Imatinib, by making them sensitive to TRAIL, a well-studied anti-cancer agent. In previous study, a known anticancer agent, Hydroxychavicol, has been shown to have cytotoxic effect against K562 cells which were sensitive to Imatinib. So, we tested this compound to imatinib resistant K562 cells. In imatinib resistant K562 cells, XIAP, FLIP have emerged as targets by hydroxychavicol to sensitize cells to TRAIL mediated apoptosis via ROS, especially H_2O_2 dependant manner. ROS decreases XIAP, FLIP in imatinib sensitive K562(S) and imatinib resistant K562(R) both. In K562(R) cells, JNK and ERK differentially regulate FLIP and XIAP, respectively. ROS mediated activated ERK decreases Akt phosphorylation, Akt binding to XIAP and stabilization of XIAP. ROS activated JNK increases ITCH expression thereby ITCH binds to FLIP and cause FLIP degradation. So, ROS degrades FLIP, XIAP by ubiquitin mediated pathway. But degradation pathway of these antiapoptotic proteins has identified different in K562(S) cells. In K562(S) cells, ROS degrades XIAP, FLIP by lysosomal degradation pathway. ROS activated JNK causes lysosomal degradation of XIAP, FLIP both. In K562(S) cells, ERK remained activated that cause Akt phosphorylation that increase XIAP, FLIP upregulation in absence of ROS. Activated ERK give antiapoptotic signaling in K562(S) cells. ROS treated K562(S) cells, ERK is dephosphorylated so Akt remains dephosphorylate so, XIAP, FLIP lysosomal degradation is occurred. Thus our findings may enlighten a differential signalling pathway between Imatinib resistant and sensitive cells which may be targeted for the treatment of later stages of the disease of CML patients.

Introduction: Chronic Myeloid leukemia is diseases of myeloproliferative disorder of pluripotent stem cells which results from reciprocal translocation of t(9;22)(q34;q11) and formation of constitutively active Philadelphia Chromosome . In Philadelphia chromosome, fused BCR-ABL gene encodes constitutively active tyrosine kinase protein, BCR-ABL [1].

Imatinib is potent inhibitor of tyrosine kinase ABL, is first-line drug for the treatment of CML [2]. Generation of resistance against imatinib is occurred due to BCR/ABL amplification and over-expression, BCR/ABL kinase domain mutations and clonal evolution with activation of additional oncogenic pathways.

Apoptosis is programme cell death. Apoptosis is important for elimination of infected or damaged cells that interfere with a normal function. The loss of apoptotic control contributes cancer cells to be immortal [3-5]. Regulation of apoptosis, both Intrinsic and Extrinsic, occurs at various levels. FLIP and XIAP are two of the well-established regulators of apoptosis.

FLIP is a pro-caspase 8 mimetic. It contains two DEDs like the initiator caspase. It is anti-apoptotic protein due to the absence of caspase like activity, due to the lack of the catalytic cysteine residue that is present in pro-caspase 8 [6-9]. FLIP competes with caspase-8 to prevent BID activation and inhibits intrinsic apoptotic pathway. FLIP upregulation also blocks TRAIL mediated apoptosis because the recruitment of FLIP to the DISC instead of procaspase-8 or -10 can block caspase activation [10].

XIAP belongs to the Inhibitor of Apoptosis Protein (IAP) family. It contains 1-3 baculovirus IAP repeat (BIR) domains, a carboxyterminal RING (really interesting new gene) zinc-finger domain which possess E3 ubiquitin ligase activity[11,12]. It inhibits active caspase 9 through its BIR3 domain and inhibits active caspase 3,7 through its linker BIR2 domain. XIAP has ubiquitin ligase activity, ubiquitinates the caspase 3 and degrade by proteosome [13-16].

XIAP and FLIP are promising targets for therapeutic involution because these proteins are overexpressed in many cancer cells including haematological malignancy and linked with treatment failure [17,18]. Elevated expression of these antiapoptotic proteins resists agonist antibodies that stimulate Death Receptor (DR) mediated apoptosis [19]. Overexpression of XIAP, FLIP activate different type of signal transduction pathways that enhance cell proliferation, promote cell survival [20,21]. These two actions promote unrestrained tumour growth, resilience that make XIAP, FLIP, promising target for killing cancer cells.

Objectives of my Thesis work:

1. Identification of Inhibitor of apoptotic protein as a target of induction of apoptosis in imatinib resistant CML cells.

2. To identify the modifications of IAPs and signalling mechanisms involved in the modification
3. To check the key findings in imatinib resistant cells from objective I and objective II in imatinib sensitive cells.

Material and Method

Materials: Hydrogen peroxide was purchased from Merck Life Science Pvt. Ltd . Propidium iodide, Imatinib mesylate were bought from Sigma-Aldrich and DR5 blocking chimera rhTRAIL was bought from R&D Systems Inc. (Minneapolis, Minnesota, USA). MTT and NAC (N-acetyl-L-cysteine), 2′,7′- Dichlorofluorescin diacetate were bought from Calbiochem. iScript™ Reverse Transcription Supermix ,SsoFast™ Evagreen® Supermix were purchased from BIO RAD (USA). FITC Annexin V was taken from BD Pharmingen. The antibodies against caspase-8, caspase-9, caspase-3, PARP, DR5, XIAP, c-FLIP, Bcl2, survivin, CIAP, BAX, tBID, Akt, pAkt, PI3K, pPI3K, ERK and JNK, pJNK pERK1/2, ITCH, anti-mouse IgG HRP-linked antibody and anti-Rabbit IgG HRP-linked antibody, Control siRNA, ERK1/2 siRNA, JNK siRNA were bought from Cell Signaling Technology (Denver, Massachusetts,USA). Anti-γ-actin antibody used as a control was bought from Biobharati Life Science (India). pcDNA3 Flag JNK2a1 was a gift from Roget Davis (Addgene plasmid # 13754; http://n2t.net/addgene:13754; RRID: Addgene_13754) and pcDNA3-HA-ERK2 WT was a gift from John Blenis (Addgene plasmid # 8974; http://n2t.net/addgene:8974; RRID: Addgene_8974). SureBeads™ Protein G Magnetic Beads was bought from BIO RAD (USA). JNK inhibitor SP600125, ERK inhibitor PD98059, p38 inhibitor SB203580 were purchased from Abcam (Cambridge, MA, USA). Fetal Bovine Serum (FBS) Standard was purchased from Gibco®.Life Technologies, USA.

Methods:

Cell lines and culture condition: Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum and 0.1% ampicillin and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with continuous flow of 5% carbon dioxide (CO2).

Development of imatinib-resistant K562 cells: Imatinib was added to the cell culture medium in a graded increasing dose, and the viable cells were selected and placed in the medium with next higher doses of imatinib. In this way, final imatinib concentration was

reached at 1.5 μg/ml, where 100% cells were made viable. These cells were designated as K562(R).

Annexin V/PI binding assay: 1.0×10^5 cells were washed with PBS twice and annexin V binding buffer was added into it. Then annexin V/FITC was added in it and kept for 30 mins at dark place. Next 50μg/ml PI was added and flow cytometry was done mixing the cells with 400μl of 1X Annexin V binding buffer. Analysis was done in a flowcytometer (BD accurie) and BD C6 software.

Real time PCR: Using Trizol reagent the total RNA was isolated from appropriately treated cells according to the manufacturer's instructions. cDNA was prepared from isolated total RNA using iScriptTM Reverse Transcription Supermix for RT-qPCR (BIO RAD). Real-time PCR was done with SsoFastTM Evagreen Supermix (BIO RAD). 40 cycles were run for each round of PCR for total saturation and the Cq values were considered for further analysis. All RT-qPCR for complete experiments were independently repeated at least three times. For graphical representation of the gene expression data, Actin was used as house-keeping gene and the mRNA levels were normalized accordingly.

Western Blot: Cells were washed in ice-cold PBS and lysed with 1X RIPA (CST, Cat no 9806S) and protease inhibitor cocktails. Lysate are collected by centrifugation at 15000 rpm for 15 min. Proteins samples were boiled with Laemmli buffer at 95 °C for three minutes and subjected to SDS PAGE. Then Proteins were transferred to PVDF membrane. Then the membranes were blocked with 5% BSA (for phospho-epitope) or 5% not fat milk diluted in TBST for 1 hr. Proteins were detected using the respective antibodies corresponding HRP linked secondary antibodies were used for detection. Bands were developed using BIO RAD Clarity™ Western ECL substrate. Image were developed by BIO RAD ChemiDoc™ MP Imaging System.

Knock down assay: 2×10^6 cells/well were kept in overnight without serum and antibiotics and transfected with siRNA oligo using Lipofectamine 2000 (Invitrogen) and RPMI (without serum and antibiotic) medium, according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose–response studies. Silencing was examined 48 h after transfection.

Gene Overexpression: To ectopically overexpress JNK and ERK gene, cells were transfected with pcDNA3 Flag JNK2a1 (Addgene plasmid # 13754) and pcDNA3-HA-ERK2 WT (Addgene plasmid # 8974) using Lipofectamine 2000 according to the manufacturer's protocol. Cells were treated with H_2O_2 24 hr post transfection and overexpression was checked by western blot.

Objective I: Identification of Inhibitor of apoptotic protein as a target of induction of apoptosis in imatinib resistant CML cells.

Imatinib is widely accepted drug for the treatment of CML. Development of resistance of this drug is major problem for the treatment of CML. Alternative therapeutic approach might prove effective treatment for the imatinib resistance patients with CML.TRAIL (The tumor necrosis factor-related apoptosis-inducing ligand) could be this alternative treatment choice because TRAIL acts as promising anticancer drug as TRAIL shows high toxicity toward cancer cells and less toxicity toward the normal cells [22]. However K562(R) cells are TRAIL resistant. Combination therapeutic approach involving TRAIL along with any agent that induces K562(R) to TRAIL mediated apoptosis that could be an interesting concept in the treatment of imatinib resistant CML patients [23]. In this objective we use Hydroxychavicol which is a phenolic compound of Piper betel leaves. Previous study suggested that Hydroxychavicol has anticarcinogenic activity against CML [24,25,26].

Hydroxychavicol sensitizes K562(S) cells to drug induced apoptosis. We use Hydroxychavicol to extend that work into resistant cells. TRAIL has been shown to induce Extrinsic apoptotic pathway (27-32). However, K562(R) cells were not sensitive to TRAIL.Therefore; we use Hydroxychavicol to sensitize K562(R) cells to TRAIL induced apoptosis.

Result:

Hydroxychavicol sensitizes imatinib-resistant CML cell lines to TRAIL-induced apoptosis

To check resistance of this K562 cells to imatinib, K562 cells were treated 1.5 μg/ml of Imatinib. Annexin V/PI binding assay showed that imatinib induced almost 37% cell death in normal untreated K562 cells whereas only 10% cell death was observed in case of the imatinib-resistant K562(R) cell (Fig1A).

When two cell lines (both imatinib sensitive and imatinib resistant) were incubated with various doses of TRAIL, annexin V/PI binding assay showed very little or no cell death in K562(R) cells by TRAIL (Fig1B). Imatinib resistant K562 cells are used in subsequent experiments.

To confirm role of Hydroxychavicol on TRAIL mediated apoptosis, K562 (R) cells were treated with Hydroxychavicol for 6 hrs. , then incubated with 200ng/ml TRAIL for 18 hrs. Annexin V/PI binding assay and MTT assay confirmed that Hydroxychavicol and TRAIL alone induced only 5–10% cell death. Cytotoxicity increased significantly after 24 hrs when both were used in combination (Fig 1C,1D). Next, caspase cleavage assay was done to confirm apoptosis enhancement by TRAIL. K562(R) cells were treated with 4 μmol/l and 6 μmol/l of Hydroxychavicol alone or in combination of 200 ng/ml TRAIL for 18 h. Result showed that combinatory treatment significantly enhanced cleavage of caspase 3,8, PARP, no caspase 9 cleavage is occurred. Pro caspase 8 cleavage is occurred, It suggested that extrinsic pathway is involved in HCH mediated TRAIL induced apoptosis (Fig1e).

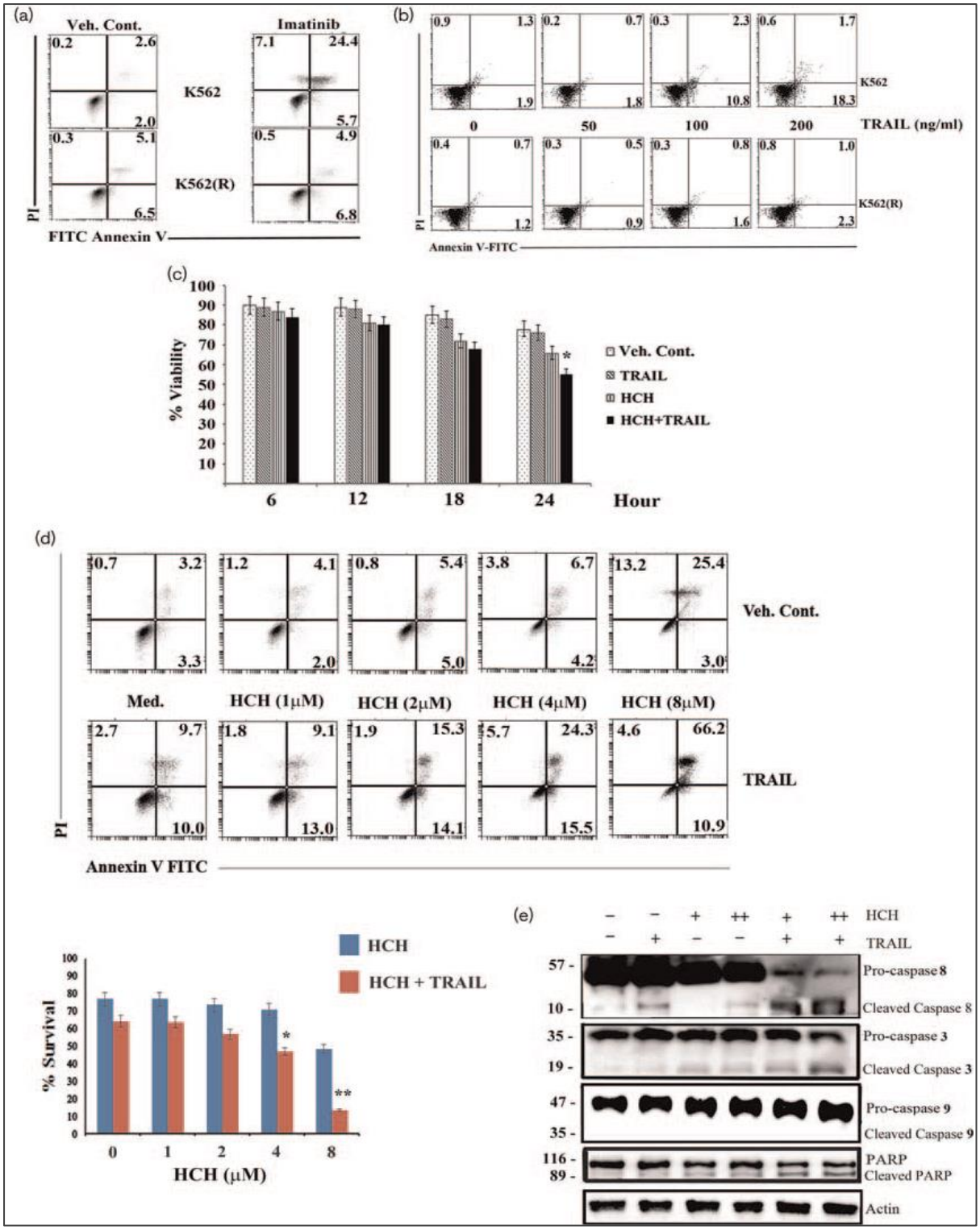

(a)
Veh. Cont.
Imatinib
K562
K562(R)
PI
FITC Annexin V
(b)
K562
K562(R)
TRAIL (ng/ml)
0
50
100
200
PI
Annexin V-FITC
(c)
% Viability
Veh. Cont.
TRAIL
HCH
HCH+TRAIL
Hour
(d)
Veh. Cont.
TRAIL
Med.
HCH (1μM)
HCH (2μM)
HCH (4μM)
HCH (8μM)
PI
Annexin V FITC
(e)
HCH
TRAIL
Pro-caspase 8
Cleaved Caspase 8
Pro-caspase 3
Cleaved Caspase 3
Pro-caspase 9
Cleaved Caspase 9
PARP
Cleaved PARP
Actin
% Survival
HCH
HCH + TRAIL
HCH (μM)

Figure 1

Fig1: Hydroxychavicol enhances the sensitivity of imatinib-resistant K562(R) cells to TRAIL. a) Imatinib sensitive K562 and Imatinib resistant K562 (R) cells were treated with 1.5µg/ml imatinib. Cells were analysed by annexinV/PI binding assay by flow cytometry b) K562(R) and sensitive K562 cell were treated with indicated doses of TRAIL, and annexin V/PI binding assay was performed in flowcytometer. C) K562(R) cells were treated with 6µmol/l of HCH and 200 ng/ml of TRAIL. After 24 hrs, cell viability was measured by MTT assay. Data represent mean ±SD of three independent experiments (*P<0.05). d) K562(R) cells were treated with indicated doses of HCH and 200 ng/ml of TRAIL. Annexin V/PI binding assay was performed in flowcytometer. Data represent mean ±SD of three independent experiments (*P<0.05). e) K562 (R) cells were treated with Hydroxychavicol and TRAIL (tumour necrosis factor-related apoptosis-inducing ligand). Whole cells lysate were subjected for Western blot analysis using antibody against pro caspases-8, 3, 9 and PARP, and actin was used as a loading control.

Altered signaling downstream to TRAIL receptors DR4 and DR5 plays a key role in Hydroxychavicol-mediated TRAIL sensitization in K562(R) cells:

As TRAIL binds to death receptor and induces apoptosis. We wanted to confirm the involvement of TRAIL-apoptosis pathway by Hydroxychavicol and TRAIL. To do that, Cells were preincubated with DR5 blocking chimera antibody for antagonizing TRAIL binding for 2hrs, then treated with Hydroxychavicol and TRAIL either alone or in combination. The flowcytometer analysis showed that DR5 blocking chimera antibody significantly reversed TRAIL-mediated apoptosis (Fig2a). This indicated that TRAIL mediated extrinsic apoptotic pathway was involved in above combinatory effect. One possibility might be that whether Hydroxychavicol increased DR4 and DR5 expression. Cells were treated with Hydroxychavicol at various time points. Result indicated that there was no increase in DR4 and DR5 at 18 hr (Fig2b). Data indicated that DR4, DR5 remains unaltered at various doses of Hydroxychavicol (Fig2c). DR4, DR5 expressions at mRNA level remain unaltered at various doses of HCH (Fig2d). DCR1, DCR2 are both TRAIL receptors. But DCR1/DCR2 cannot induce apoptosis [33,34]. There was no change in DCR1/DCR2 at protein level (Fig2e) and mRNA level (Fig2f) after treatment of Hydroxychavicol. Our data indicated that Hydroxychavicol involved death receptor-mediated signaling pathway for induction TRAIL mediated apoptosis, while death receptor expression remains unaltered.

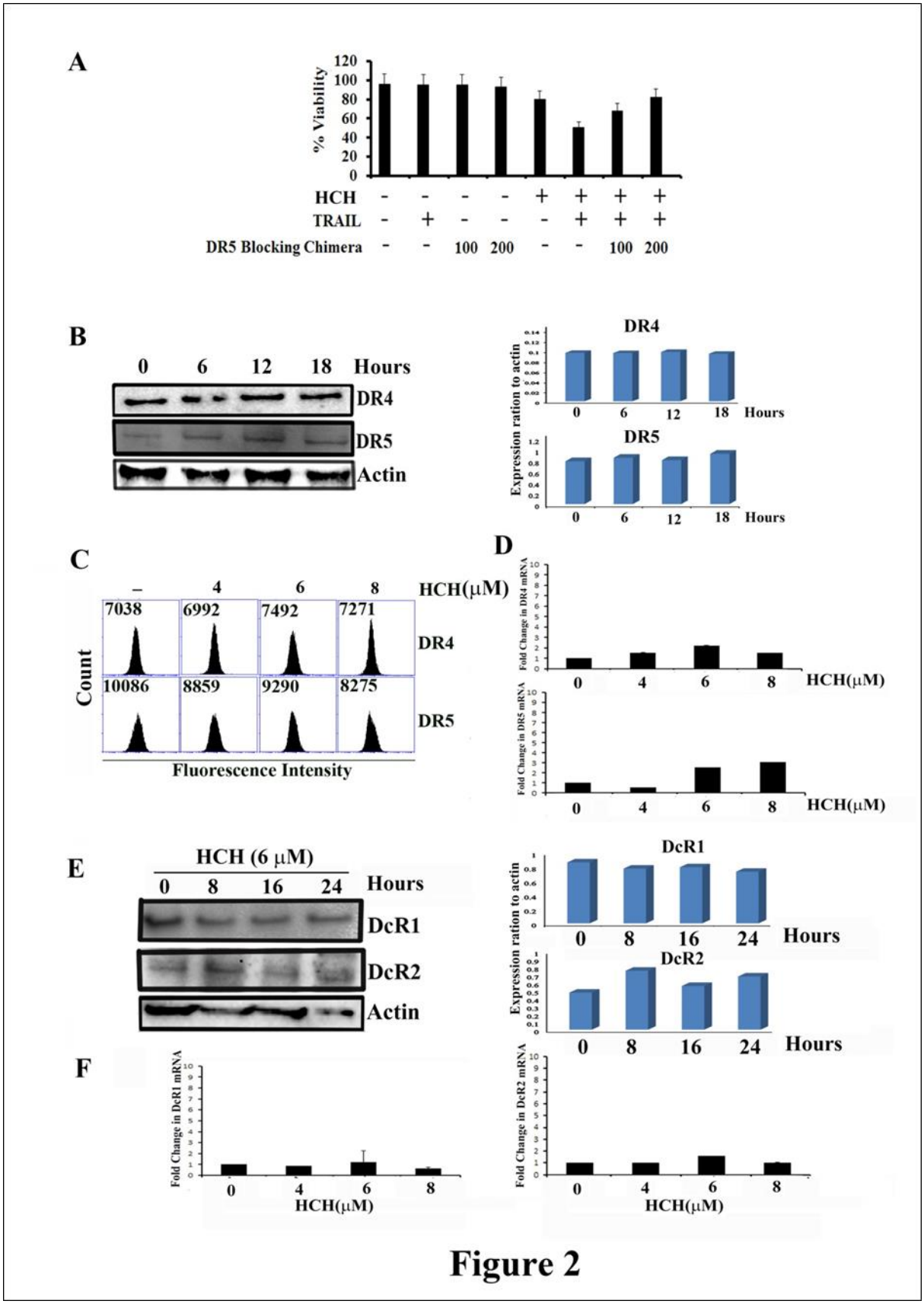

A
% Viability
120
100
80
60
40
20
0
HCH - - - - + + + +
TRAIL - + - - - + + +
DR5 Blocking Chimera - - 100 200 - - 100 200
B
0 6 12 18 Hours
DR4
DR5
Actin
DR4
Expression ration to actin
0 6 12 18 Hours
DR5
0 6 12 18 Hours
C
– 4 6 8 HCH(μM)
7038 6992 7492 7271
DR4
10086 8859 9290 8275
DR5
Count
Fluorescence Intensity
D
Fold Change in DR4 mRNA
0 4 6 8 HCH(μM)
Fold Change in DR5 mRNA
0 4 6 8 HCH(μM)
E
HCH (6 μM)
0 8 16 24 Hours
DcR1
DcR2
Actin
DcR1
Expression ration to actin
0 8 16 24 Hours
DcR2
0 8 16 24 Hours
F
Fold Change in DcR1 mRNA
0 4 6 8
HCH(μM)
Fold Change in DcR2 mRNA
0 4 6 8
HCH(μM)

Figure 2

Fig2: Extrinsic apoptotic pathway is involved in TRAIL mediated apoptosis without increase expression of death receptor. (a) K562(R) cells were pretreated with DR5 blocking chimera antibody and then treated with the combination of HCH and TRAIL. Cell viability assay was performed by MTT assay. Data represent mean ±SD of three independent experiments (*P< 0.05). (b) K562(R) cells were treated with various doses of Hydroxychavicol. Whole cell lysate were subjected to western blot analysis for checking DR4/DR5 at protein level. β-actin acts as a loading control. (c) K562 cells were treated with various doses of Hydroxychavicol for 14 hrs. DR4 and DR5 were checked in flowcytometer by labelling them with DR4/DR5 antibody and fluorescent conjugated secondary antibody. Data shown here area representative histogram of three independent experiments. (d) Cells were treated with various doses of Hydroxychavicol. DR4 and DR5 expression were checked by real-time PCR (n =3: data as mean ±SEM). (e) K562 (R) cells were treated Hydroxychavicol for different time point. DcR1 and DcR2 surface protein levels were checked by Western blot analysis. Here β-actin acts as a loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. (f)K562(R) cells were treated with increasing doses of Hydroxychavicol. DcR1 and DcR2 mRNA expressions were checked by real time PCR (n=3,mean ± SD).

HCH decreased FLIP and XIAP at the protein level

Overexpression of antiapoptotic genes are most commonly reported mechanism for apoptosis evasion [35-39]. To check whether any alteration of these proteins could be responsible here. Cells were treated with Hydroxychavicol at different doses at 20 hrs. Result showed that XIAP and FLIP were decreased at protein level. Bcl2 and cIAP levels remains unaltered with increasing doses of HCH (Fig3a). There was no any alternation of FLIP, cIAP, XIAP at mRNA level (Fig3b). To understand the molecular mechanism behind FLIP, XIAP downregulation, cells were treated with MG132 (proteosomal inhibitor) before incubation with Hydroxychavicol, data showed that significant inhibition of FLIP and XIAP downregulation by MG132 (Fig3c). So, XIAP, FLIP degradation is occurred via proteosomal mediated pathway. To check XIAP, FLIP downregulation played a role in apoptosis, cells were knocked down with XIAPsiRNA and FLIPsiRNA before incubation with TRAIL. This result indicated that knocking down of XIAP and FLIP enhanced apoptosis as compared with control siRNA (fig3d).

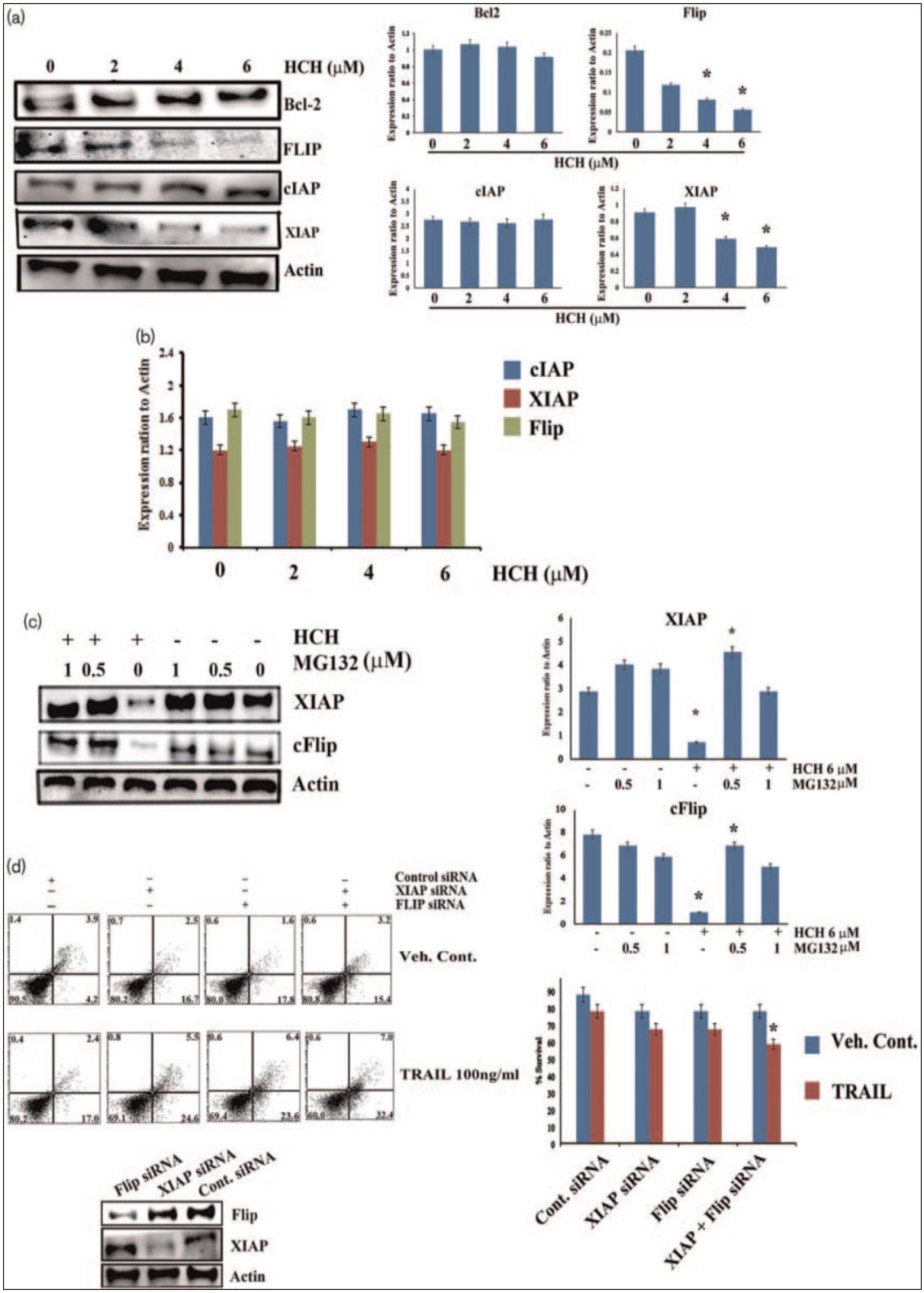

(a)
0 2 4 6 HCH (μM)
Bcl-2
FLIP
cIAP
XIAP
Actin
Bcl2
Flip
cIAP
XIAP
Expression ratio to Actin
HCH (μM)
(b)
Expression ration to Actin
cIAP
XIAP
Flip
HCH (μM)
(c)
HCH
MG132 (μM)
1 0.5 0 1 0.5 0
XIAP
cFlip
Actin
HCH 6 μM
MG132μM
(d)
Control siRNA
XIAP siRNA
FLIP siRNA
Veh. Cont.
TRAIL 100ng/ml
Flip siRNA
XIAP siRNA
Cont. siRNA
Flip
XIAP
Actin
% Survival
Veh. Cont.
TRAIL
Cont. siRNA
XIAP siRNA
Flip siRNA
XIAP + Flip siRNA

Figure 3

Fig 3: XIAP and FLIP downregulation is involved in TRAIL mediated apoptosis. (a) K562 (R) cells were treated with 6 μmol/l HCH for 18 h and whole cell lysates were subjected to Western blot analysis. Data shown here is a representative Western blot of three independent experiments. On the right, data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ±SD of three independent experiments (*P< 0.05). (b) Levels of endogenous c-FLIP, cIAP, and XIAP mRNA were assessed by real-time PCR after treatment with increasing doses of Hydroxychavicol for 14 hrs. and graphically represented as expression ratio to actin as mean ±SD of three independent experiments.(c) K562(R) cells were preincubated with MG132 and then treated with Hydroxychavicol for 16 hrs. Whole cell lysate were subjected to western blot. The Western blot data are a representative one from three independent experiments. Data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments (*P<0.05). (d) K562(R) cells were transfected with control siRNA, FLIPsi RNA, XIAPsi RNA for 48 h and then treated with 200 ng/ml TRAIL. Apoptosis assay was done by annexin V/PI binding assay by flowcytometer. Data represent mean ±SD of three independent experiments (*P<0.05). At bottom panel, XIAP and FLIP level was checked after knockdown of cell with XIAP siRNA and FLIP siRNA respectively.

ROS played a major role in Hydroxychavicol -mediated TRAIL sensitization

It is reported that Hydroxychavicol increases the intracellular ROS level [29,30]. When cells were preincubated with ROS scavenger NAC then treated with Hydroxychavicol and TRAIL, NAC reversed TRAIL mediated apoptosis. This indicated that ROS involvement in this process (Fig4a). ROS generation assay showed that ROS was increased from 15 min, reached peck level at 30 min after treatment of Hydroxychavicol (Fig4b). TRAIL did not change ROS level when TRAIL incubated with various concentrations alone (Fig4c). ROS level was increased at various concentration of Hydroxychavicol, keeping TRAIL concentration fixed (Fig4d). It confirmed that TRAIL has no additive effect in ROS generation.

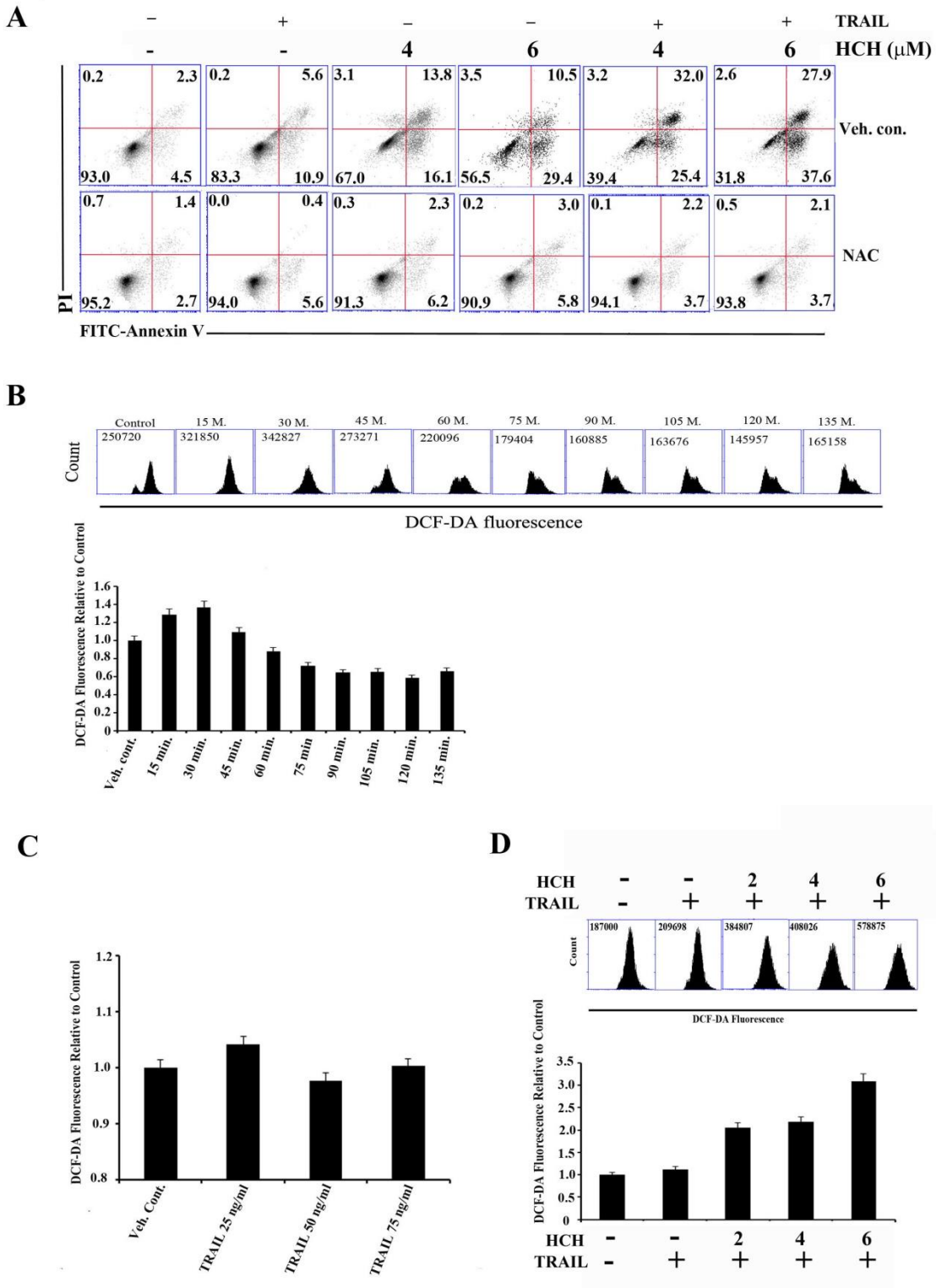

A
TRAIL
HCH (μM)
Veh. con.
NAC
PI
FITC-Annexin V
B
Control
15 M.
30 M.
45 M.
60 M.
75 M.
90 M.
105 M.
120 M.
135 M.
Count
DCF-DA fluorescence
DCF-DA Fluorescence Relative to Control
Veh. cont.
15 min.
30 min.
45 min.
60 min.
75 min
90 min.
105 min.
120 min.
135 min.
C
DCF-DA Fluorescence Relative to Control
Veh. Cont.
TRAIL 25 ng/ml
TRAIL 50 ng/ml
TRAIL 75 ng/ml
D
HCH
TRAIL
Count
DCF-DA Fluorescence
DCF-DA Fluorescence Relative to Control

Figure 4

Fig4: HCH induced TRAIL sensitization of K562(R) cell is ROS dependent: (a) K562 (R) cells were preincubated with ROS scanverger, NAC, and treated with Hydroxychavicol and TRAIL for 24 hrs. Flow cytometry analysis was done after staining with Annexin V and PI . Results were representative of three independent experiments. (b) K562(R) cell line was treated with HCH (6 μmol/l), and then cells were stained with DCFDA for measuring RS level in Flow cytometry. The histograms are representative data of three independent experiments. Below is the bar diagram of mean ± SD of three independent experiments.(c) K562(R) cell line was treated with various doses of TRAIL and ROS level was measured in flowcytometer after incubation with DCFDA. The bar diagram represents mean ±SD of three independent experiments. (d) K562(R) cells were incubated with 200 ng/ml of TRAIL, and different doses of Hydroxychavicol, and DCFDA fluorescence was measured in flowcytometer. Bar diagram of mean ±SD of three independent experiments..

Downregulation of XIAP and FLIP happens in ROS dependant manner

To check whether Hydroxychavicol mediated XIAP, FLIP downregulation was ROS mediated, cells were preincubated with NAC then treated with Hydroxychavicol. Result indicated that NAC reversed HCH mediated XIAP, FLIP downregulation (Fig5a). If ROS have role in XIAP, FLIP downregulation, adding ROS externally should show similar effect. H_2O_2 downregulate XIAP, FLIP after treatment with different doses of H_2O_2.(Fig5b). If ROS is responsible for this downregulation, then ROS itself should sensitize K562(R) cells to TRAIL. TRAIL was applied on H_2O_2-pretreated cells. Data indicated that TRAIL enhanced apoptosis in H_2O_2-treated cells (Fig5c). The role of ROS in Hydroxychavicol-mediated TRAIL-induced apoptosis was confirmed by TRAIL-mediated caspases-8, 3, PARP cleavage which was reversed by NAC (Fig5d).

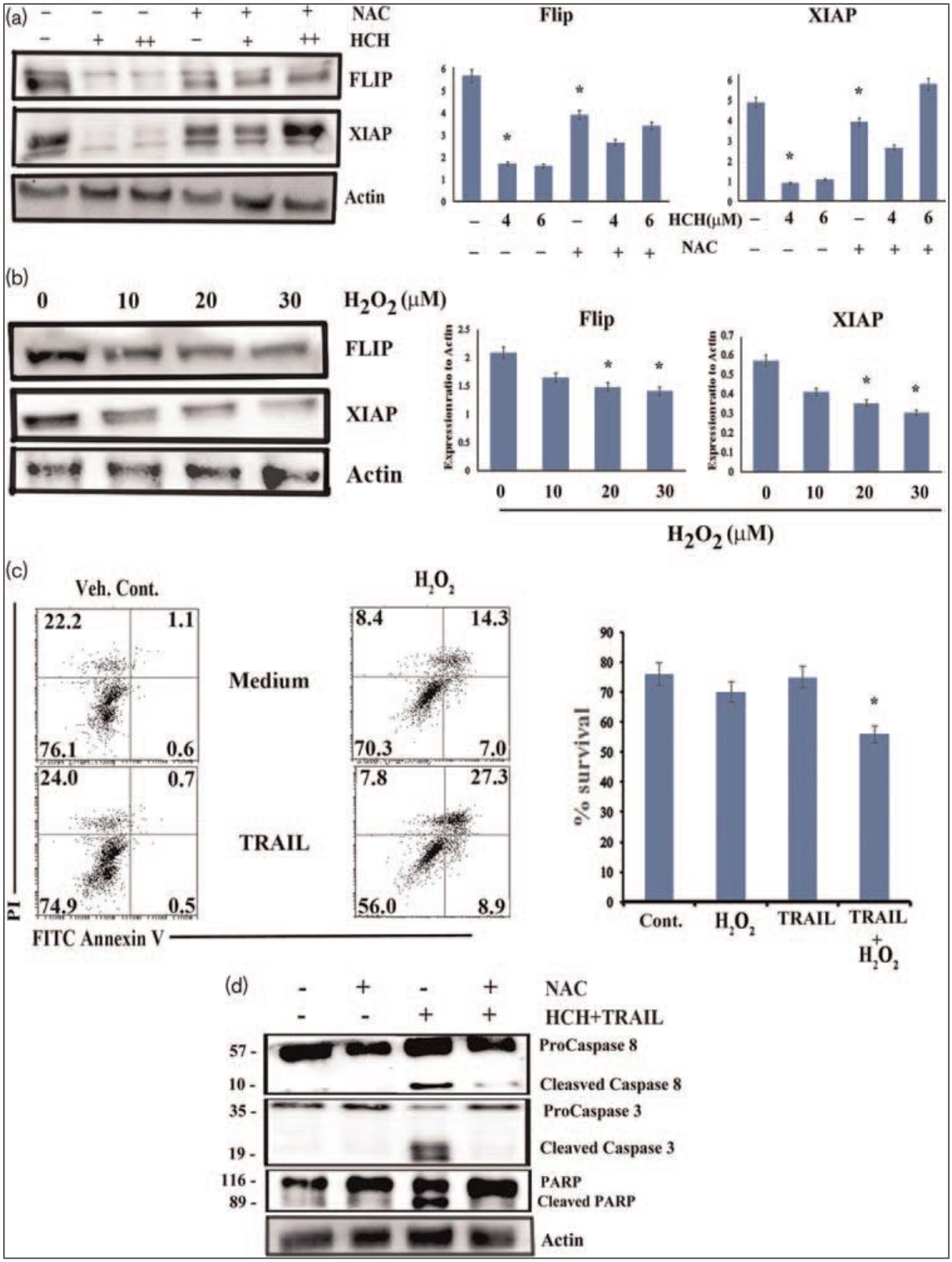

(a)
NAC
HCH
FLIP
XIAP
Actin
Flip
XIAP
HCH(μM)
NAC
(b)
H_2O_2 (μM)
FLIP
XIAP
Actin
Flip
XIAP
Expressionratio to Actin
H_2O_2 (μM)
(c)
Veh. Cont.
H_2O_2
22.2
1.1
76.1
0.6
8.4
14.3
70.3
7.0
Medium
24.0
0.7
74.9
0.5
7.8
27.3
56.0
8.9
TRAIL
PI
FITC Annexin V
% survival
Cont.
H_2O_2
TRAIL
TRAIL + H_2O_2
(d)
NAC
HCH+TRAIL
57 -
10 -
35 -
19 -
116 -
89 -
ProCaspase 8
Cleasved Caspase 8
ProCaspase 3
Cleaved Caspase 3
PARP
Cleaved PARP
Actin

Figure 5

Fig5: ROS plays a role in Hydroxychavicol mediated XIAP, FLIP downregulation: (a) K562(R) cells were preincubated with NAC, then treated with Hydroxychavicol. Whole cell lysate were subjected to western blot analysis to measure XIAP, FLIP protein level. Data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments (*P<0.05). (b) Cells were treated with indicated dose of H_2O_2 for 16 hrs. FLIP and XIAP protein level were checked by western blot. Data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ±SD of three independent experiments (*P<0.05).(c) K562 (R) cells were treated with TRAIL and H_2O_2. Apoptosis assay was performed by annexin V/PI binding assay. Data are a representative dot plot of three independent experiments. The data are also represented as Bar graph which denotes mean ±SD of viable cells [100– (upper left upper right lower right quadrant percent)] from three independent experiments (*P< 0.05). (d) K562(R) cells were preincubated with NAC, and treated with HCH and TRAIL. Whole cell lysate were subjected to western blot analysis for checking the protein level of pro caspase-8, 3, and PARP. Data are a representative blot of three independent experiments.

It is reported that IAPs can be targeted by several agent. From objective I, it is confirm that IAPs can be targeted by Hydroxychavicol thereby TRAIL mediated apoptosis can be induced in imatinib resistant CML cells.

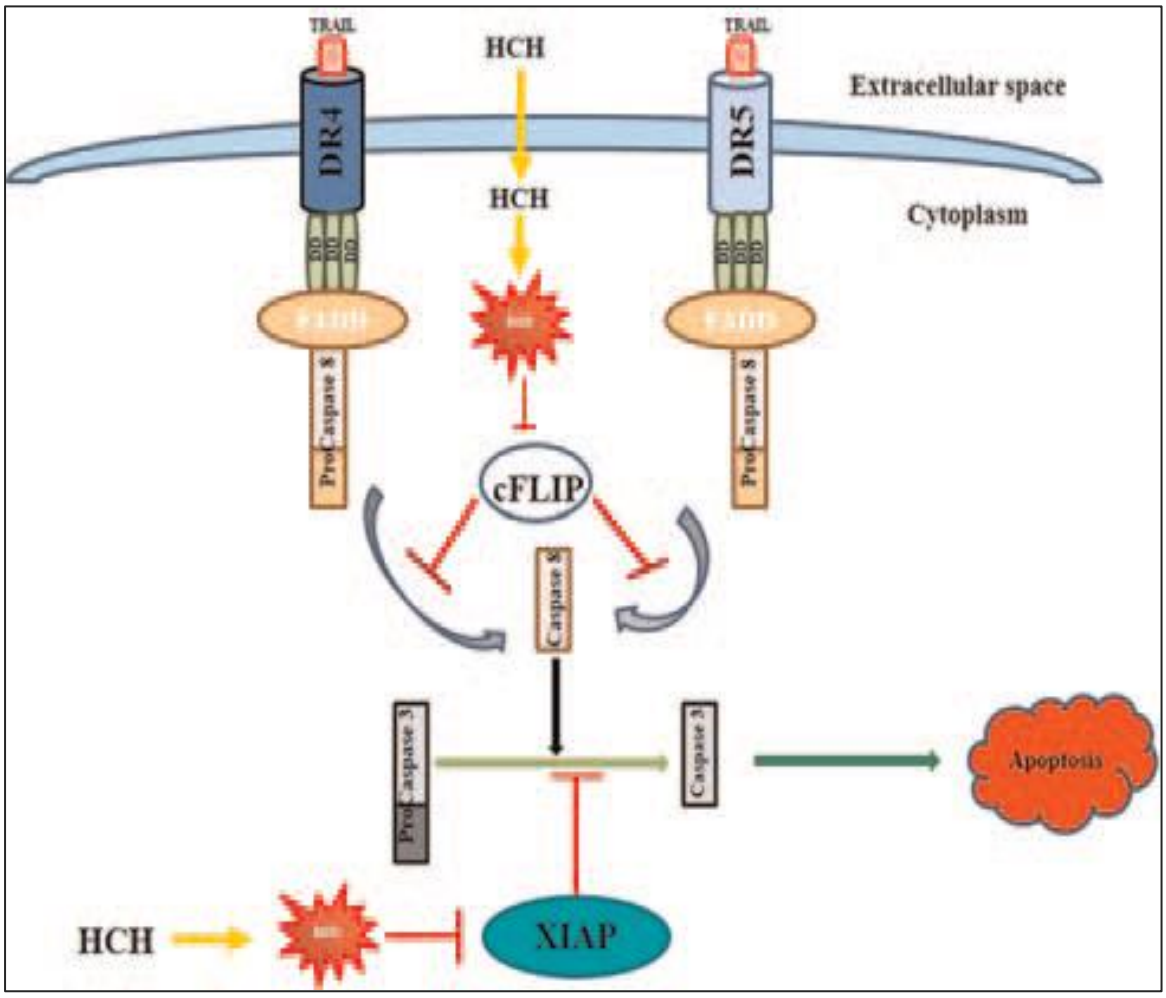


Figure 6: The mechanism of action of HCH in the sensitization of TRAIL in K562(R) cells. HCH sensitizes K562-resistant CML cells to TRAIL-induced apoptosis through downregulation of XIAP and FLIP via ROS generation.

Objective II: To identify the modifications of IAPs and signalling mechanisms involved in the modification

In objective I, we have reported that downregulation of XIAP, FLIP become a strong strategy for TRAIL mediated apoptosis in imatinib resistant CML. Hydroxychavicol downregulate XIAP, FLIP in ROS dependant manner, specifically H_2O_2 dependant manner. In this objective, we have examined that the signalling mechanism that is involved in H_2O_2 dependant XIAP, FLIP downregulation.

Result:

H_2O_2 downregulates XIAP and FLIP:

To check effect of ROS on XIAP, FLIP downregulation, cells were treated with different doses of H_2O_2. Result indicate that H_2O_2 dose dependently decreased FLIP, XIAP at protein level (Fig7a). XIAP, FLIP are decreased at 16 hrs and 12 hrs respectively after treatment cells with H_2O_2 at different time points (Fig7b).As cells were treated with H_2O_2, there might be possible that induction of apoptosis, reduction in cellular protein level nonspecifically. K562(R) cells, treated with different doses of H_2O_2 for 24 hours. Result showed that there was no cytotoxicity after 24 hr (Fig7c). To check RNA level of XIAP, FLIP, cells were treated with various doses of H_2O_2. Real time PCR data indicated that there was no significant increase of XIAP, FLIP at RNA level (Fig7d). When cells were pretreated with NAC followed by H_2O_2, NAC significantly reversed XIAP, FLIP downregulation (Fig7e). So, this confirmed that H_2O_2 downregulates XIAP, FLIP at protein level.

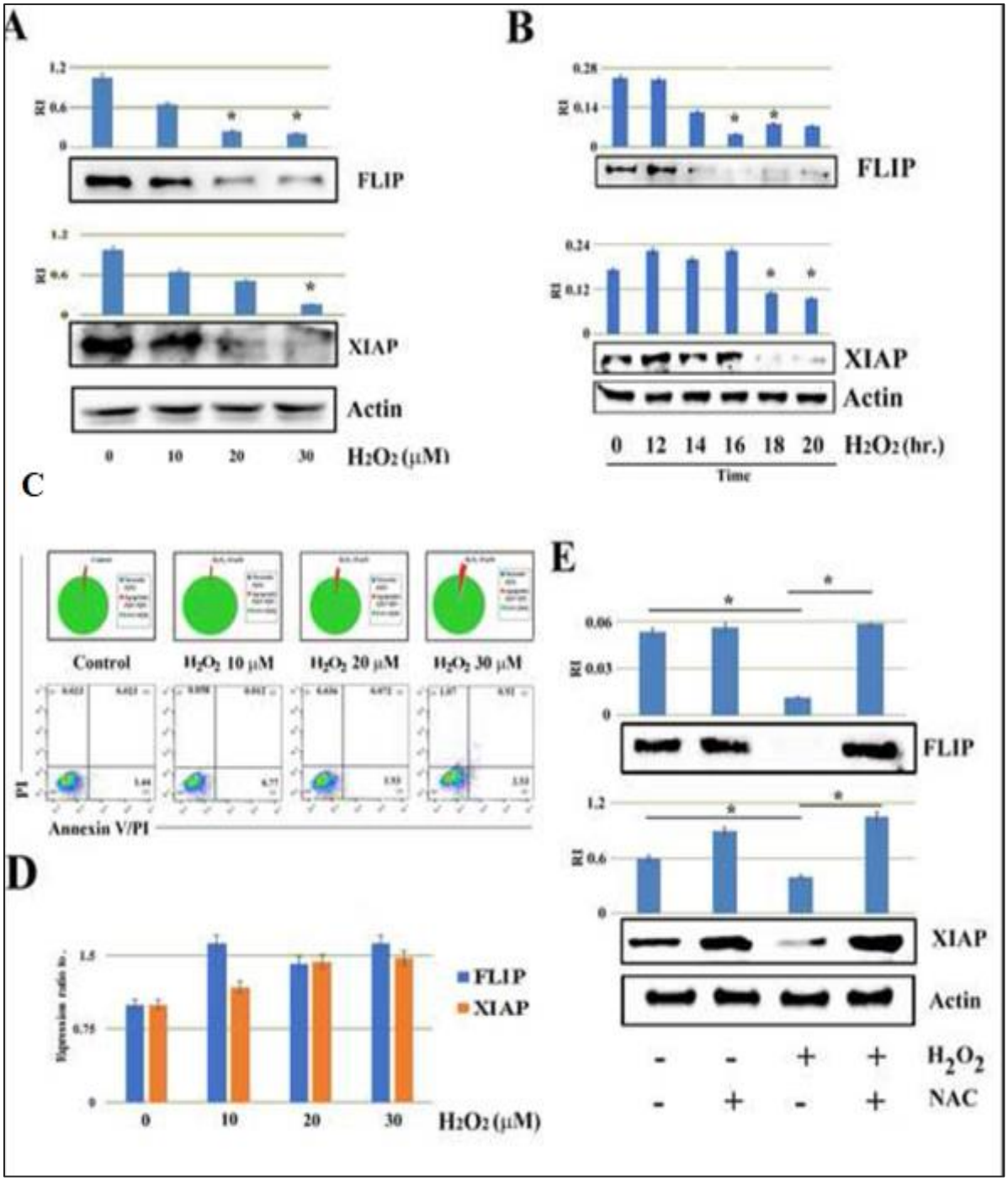

A
RI
FLIP
XIAP
Actin
0 10 20 30 H2O2 (μM)
B
RI
FLIP
XIAP
Actin
0 12 14 16 18 20 H2O2 (hr.)
Time
C
Control
H2O2 10 μM
H2O2 20 μM
H2O2 30 μM
PI
Annexin V/PI
D
Expression ratio to -
FLIP
XIAP
0 10 20 30 H2O2 (μM)
E
RI
FLIP
XIAP
Actin
- - + + H2O2
- + - + NAC

Figure 7

Fig7: ROS downregulates XIAP and FLIP. (a) K562(R) cells were treated with increasing concentration of H_2O_2. Whole cell lysate were subjected to western blot analysis for checking the protein level of XIAP, FLIP. Gamma Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05. (b) K562(R) cells were treated with 30μM H_2O_2 for indicated time points. Western blot analysis of FLIP and XIAP was performed. Gamma Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05. (c) K562(R) cells were treated with indicated doses of H_2O_2 for 18hrs. Cells were subjected to annexin V/ PI binding assay by flow cytometry. Upper panel is the graphical representation of various population in pie chart and lower panel represents representative dot plots. (d) K562(R) cells were treated with increasing concentration of H_2O_2. After 16 hrs, total RNA was extracted from cells. Cellular level of FLIP and XIAP mRNA were checked by Real time PCR. It graphically represented as fold change. Data represent mean ± SD of three independent experiments. (e) K562(R) cells were pre-incubated with 1mM NAC for 1 hr and then treated with 30μM H_2O_2 for overnight. Whole cells lysate were subjected to western blot analysis for checking protein level of XIAP, FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05.

XIAP and cFLIP degradation is proteasomal dependent

Cellular proteins level gets reduced by Lysosomal or Proteosomal degradation pathway. So, Cells were pretreated with 1.5μM proteasome inhibitor MG132, then treated with 30μM H_2O_2. Western blot analysis indicated that MG132 reverses H_2O_2 mediated degradation of XIAP, FLIP (Fig8a,8b upper-panel).Cells were preincubated with MG132 then treated with H_2O_2, cell lysate were taken after 12hrs and 16hrs, subjected with western blot analysis by anti-ubiquitin antibody after immunoprecipitation with FLIP, XIAP antibody. The result showed that MG132 increased XIAP, FLIP ubiquitination by H_2O_2 (FIG8a,8b). To check involvement of lysosomal pathway, cells were preincubated with Chloroquine, then treated with 30μM H_2O_2. Result indicated that Chloroquine didn't reverse H_2O_2 mediated XIAP and FLIP downregulation (Fig8c). These confirmed that H_2O_2 mediated downregulation of FLIP, XIAP is proteosomal mediated.

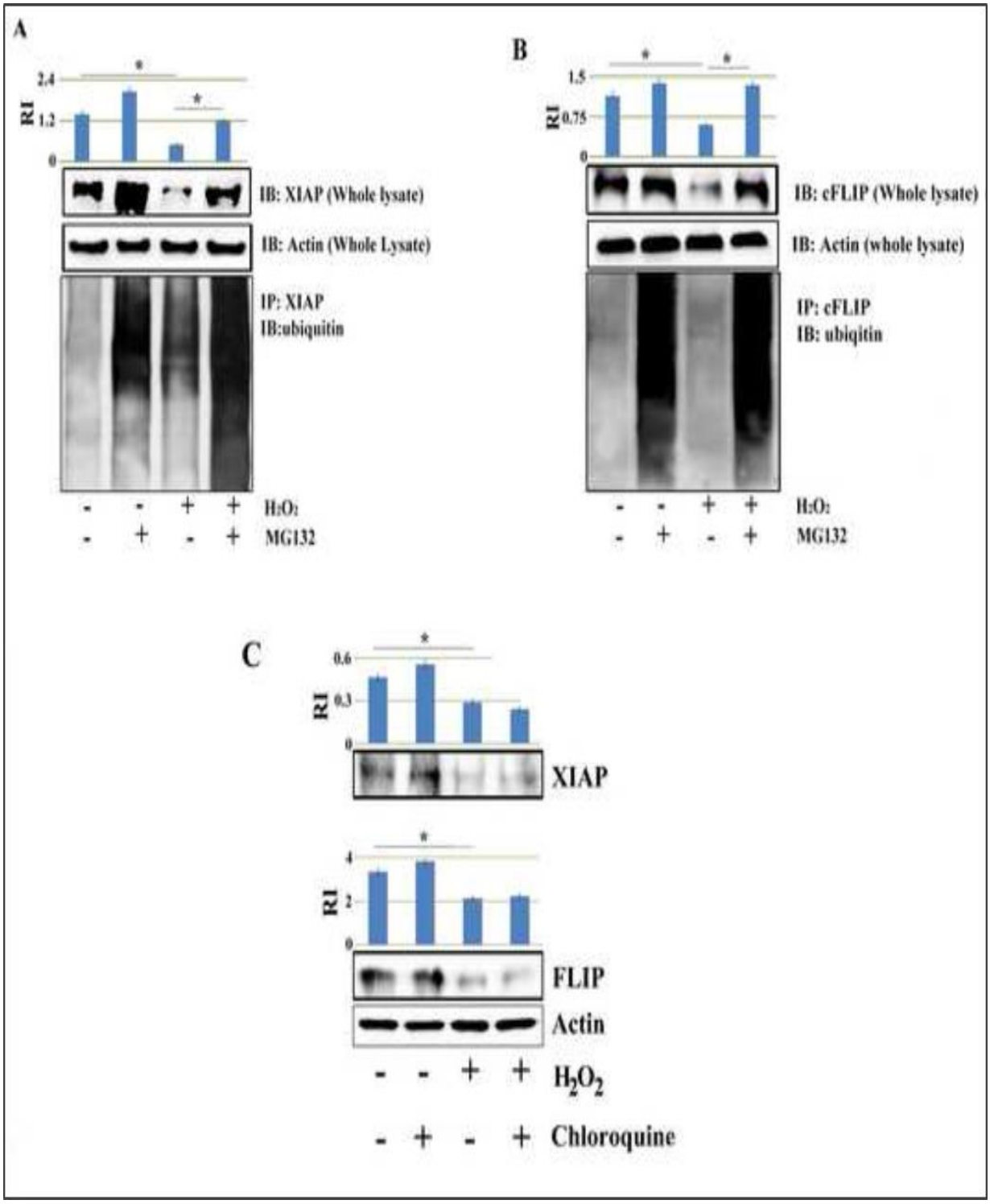

A
RI
2.4
1.2
0
IB: XIAP (Whole lysate)
IB: Actin (Whole Lysate)
IP: XIAP
IB:ubiquitin
H2O2
MG132
B
RI
1.5
0.75
0
IB: cFLIP (Whole lysate)
IB: Actin (whole lysate)
IP: cFLIP
IB: ubiqitin
H2O2
MG132
C
RI
0.6
0.3
0
XIAP
RI
4
2
0
FLIP
Actin
H2O2
Chloroquine

Figure 8

Fig 8: XIAP and cFLIP degradation is proteasomal dependent and does not involve lysosomal degradation (a) K562 (R) cells were preincubated with 2 μM of MG132 and then 30μM H_2O_2. Whole cell lysates were subjected to co-Immunoprecipitation with XIAP antibody and then western blot analysis was performed with anti-ubiquitin antibody. The upper panel shows the whole cell lysates and lower panel show the immunoprecipitates. Polyubiquitination of XIAP was happened in lower panel. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (b)) K562 (R) cells were preincubated with 2 μM of MG132 and then 30μM H_2O_2. Whole cell lysates were subjected to co-Immunoprecipitation with FLIP antibody then western blot analysis was performed with anti-ubiquitin antibody. . The upper panel shows the whole cell lysates and lower panel show the immunoprecipitates. Polyubiquitination of FLIP was happened in lower panel Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (c) K562(R) cells were preincubated with 20 μM Chloroquine and then treated with 30 μM H_2O_2. Whole cell lysates were subjected to western blot analysis with anti-FLIP and anti-XIAP antibodies. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

Activation of ERK and JNK involved in XIAP, FLIP downregulation:

MAPK pathway is major stress signalling pathway. Since ROS downregulates XIAP, FLIP. We checked whether MAPK pathway was involved in this downregulation. Cells were incubated with ERK signalling inhibitor PD98059, then treated with H_2O_2 .Result showed that PD98059 significantly reversed H_2O_2 mediated XIAP down-regulation after 18 hrs. but FLIP was not reversed by PD (Fig9a). Similar experiment was performed with JNK inhibitor SP600125. Result indicated that SP reversed H_2O_2 mediated FLIP down-regulation but not XIAP (Fig9b).Similar experiment was performed with P38 inhibitor SB202190. it didn't reverse either FLIP or XIAP(Fig9c). This confirmed that JNK, ERK were involved in FLIP, XIAP downregulation respectively. JNK, ERK show activity after phosphorylation. Cells were treated with 30 μMH_2O_2 for different time points. Result indicated that JNK phosphorylation increased around 4 hrs and peck around 16 hrs of incubation with H_2O_2. ERK phosphorylation increased around 6 hrs, decreased after 16 hrs

to its basal level (Fig9D, 9E). Protein level of JNK, ERK was unchanged after H_2O_2 treatment (Fig9F). So, H_2O_2 phosphorylate, activate ERK, JNK and these activation involved in XIAP, FLIP downregulation respectively.

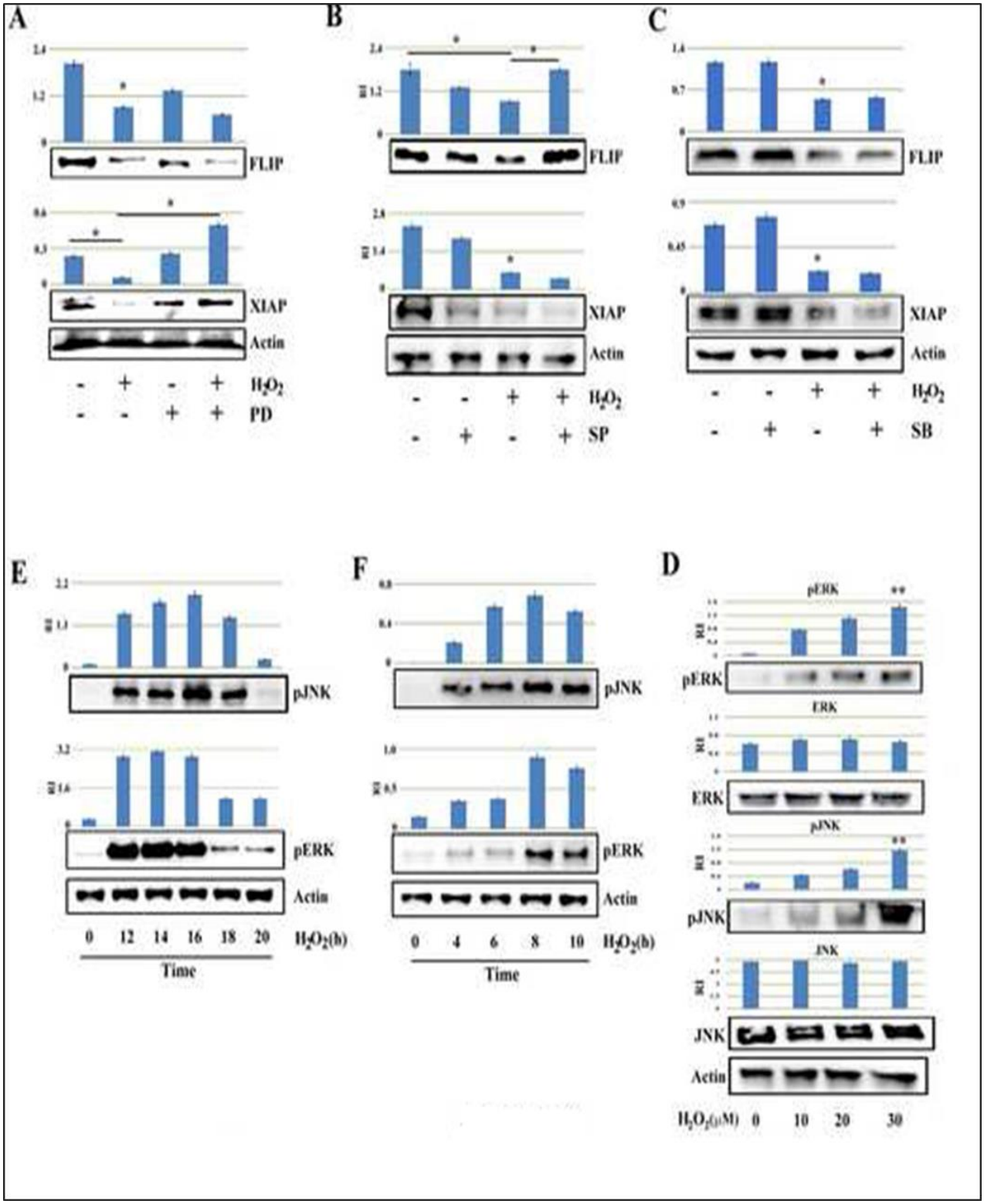


Figure 9

Fig 9: ERK and JNK are involved in regulation of XIAP and FLIP respectively in H_2O_2 treated K562(R) cells. (a) K562(R) cells were preincubated with 30 µM ERK inhibitor PD and then treated with H_2O_2 for 18 hrs. Whole cells lysate were subjected to western blot analysis for checking the protein level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (b) K562(R) cells were preincubated with 30 µM JNK inhibitor SP and then treated with H_2O_2 for 18 hrs. Whole cells lysate were subjected to western blot analysis for checking the protein level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.(c) K562(R) cells were preincubated with P38 inhibitor SB and then treated with H_2O_2 for 18 hrs. Whole cells lysate were subjected to western blot analysis for checking the protein level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (d,e) K562(R) cells were incubated with 30 µM H_2O_2 for indicated times. pJNK and pERK were checked by western blot analysis with the whole cell lysates. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. (f) K562(R) cells were incubated with indicated doses of H_2O_2 for 16 hours. Whole cell lysates were subjected to western blot analysis for checking the level of ERK, JNK, pERK, pJNK. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments**$p < 0.01$.

JNK, ERK played differential role in downregulation in cellular level of FLIP, XIAP:

To confirm role of ERK, JNK in XIAP, FLIP downregulation, K562(R) cells were treated with ERKsiRNA then treated with H_2O_2. Scrambled siRNA was used as negative control. Result indicated that H_2O_2 mediated XIAP downregulation partially reversed by ERKsiRNA treatment, but FLIP remains unchanged .ERK protein was decreased by ERKsiRNA (Fig10a).Similar knockdown assay was performed with JNKsiRNA. Result indicated that JNK knockdown reversed H_2O_2 mediated FLIP downregulation, XIAP remains unchanged. JNK decrease at protein level by JNKsiRNA (Fig10b). To confirm role of JNK, ERK, cells were transfected with pcDNA3FlagJNK2a1 plasmid and pcDNA3-HA-

ERK2WT plasmid separately, followed treatment with H_2O_2. Result indicated that JNK overexpression down-regulated FLIP more in the pcDNA3 Flag JNK2a1 containing cells than in empty vector containing cells. JNK overexpression did not downregulate XIAP by H_2O_2 (Fig10c). ERK overexpression decrease XIAP by ROS in pcDNA3-HA-ERK2WT cells than in empty vector containing cells. FLIP remained unchanged in both H_2O_2 treated cells irrespective of ERK overexpression (Fig10d). So, JNK and ERK differentially regulate FLIP and XIAP respectively.

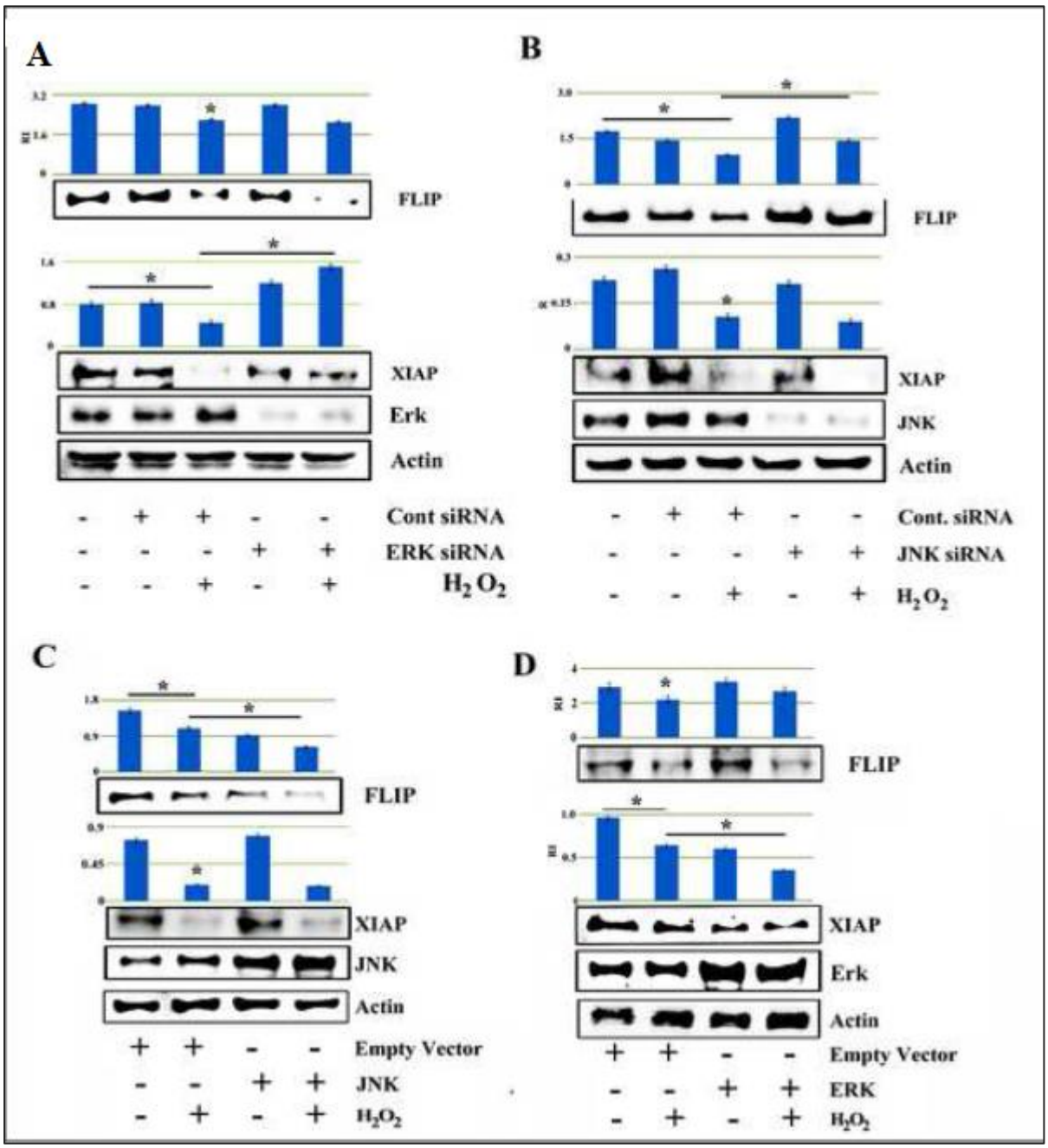


Figure 10

Fig 10: Degradation of XIAP and FLIP is ERK and XIAP mediated respectively (a) ERK was knocked down by siRNA in cells and then cells were treated with 30 μM H_2O_2.Whole cells lysate were subjected to western blot analysis for checking protein level of ERK, XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (b) JNK was knocked down by siRNA in cells and then cells were treated with 30 μM H_2O_2. .Whole cells lysate were subjected to western blot analysis for checking protein level of JNK, XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (c) K562(R) cells were transfected with either pcDNA3 Flag JNK2a1 or empty vector and then cells were treated with 30 μM H_2O_2 for 18 hrs. .Whole cells lysate were subjected to western blot analysis for checking protein level of JNK, XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (d) K562(R) cells were transfected with either pcDNA3-HA-ERK2 or empty vector and and then cells were treated with 30 μM H_2O_2 for 18 hrs. Whole cells lysate were subjected to western blot analysis for checking protein level of ERK, XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

ERK mediated PI3K and Akt dephosphorylation involved in XIAP ubiquitination

We investigated role of PI3K,Akt in XIAP downregulation. Cells were treated with PD98059, either alone or with H_2O_2. Whole cell lysate was Coimmunoprecipited with XIAP antibody, western blot was done with Akt antibody. Result indicated that amount of Akt decreased in presence of H_2O_2 alone, Akt level is reversed to level, as in control level, in presence of PD98059 (Fig11a). Result confirmed that Akt XIAP interaction is disrupted by H_2O_2, it is mediated by ERK. As PI3K, Akt were activated by phosphorylation, cells were incubated with various doses of H_2O_2, PI3K, Akt phosphorylation were decreased with increasing concentration of H_2O_2 (Fig11b). To check role of PI3K, Akt in XIAP downregulation, cells were treated with PI3K inhibitor, Wortmanin for 18 hrs. Result indicated that Wortmanin dose dependently decreased XIAP level; FLIP remained unchanged (Fig11c). Cells were pre-incubated with Wortmanin followed by H_2O_2 treatment, result showed that XIAP level decreased further than H_2O_2 alone, FLIP level similarly didn't decrease further than H_2O_2 alone (Fig11d). So, PI3K, Akt have role in XIAP downregulation, no effect on FLIP. To check role of ERK in PI3K, Akt phosphorylation, cells were treated with PD98059, then H_2O_2 treatment for 14 hrs. Result indicated that PD reversed H_2O_2-mediated dephosphorylation of PI3K and Akt (Fig11e).To confirm this data, cells were transfected with either pcDNA3-HAERK2 WT plasmid or its empty backbone, then H_2O_2 treatment, result indicated that H_2O_2 increased PI3K,Akt dephosphorylation in presence of ERK overexpression than H_2O_2 alone (Fig11f). This data confirm that H_2O_2 activated ERK, which inhibited PI3K,Akt by dephosphorylation, thus binding of Akt to XIAP was inhibited, thus degradation and ubiquitination of XIAP was occurred.

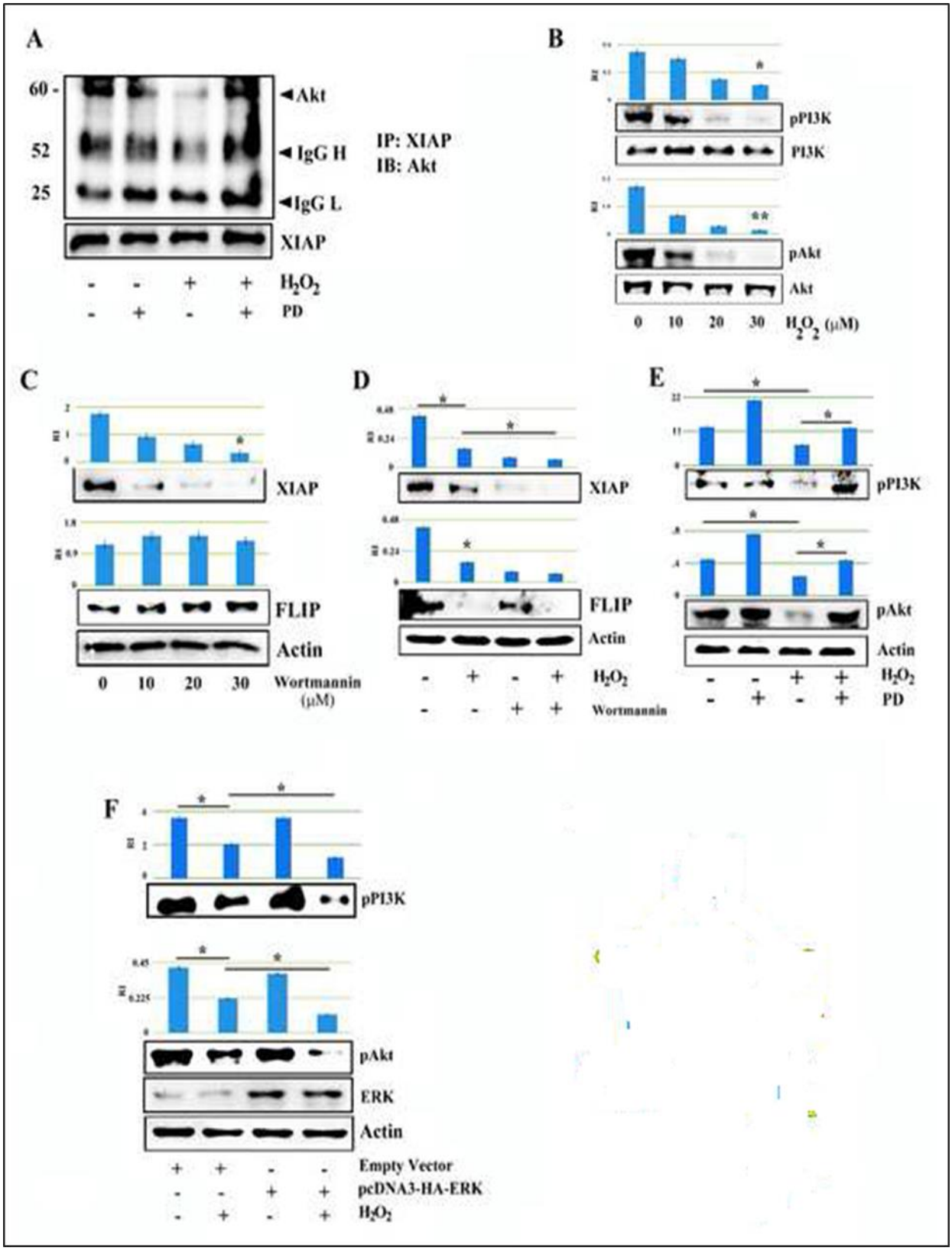

A
60 -
52
25
Akt
IgG H
IgG L
XIAP
IP: XIAP
IB: Akt
- - + + H2O2
- + - + PD
B
pPI3K
PI3K
pAkt
Akt
0 10 20 30 H2O2 (μM)
C
XIAP
FLIP
Actin
0 10 20 30 Wortmannin (μM)
D
XIAP
FLIP
Actin
- + - + H2O2
- - + + Wortmannin
E
pPI3K
pAkt
Actin
- - + + H2O2
- + - + PD
F
pPI3K
pAkt
ERK
Actin
+ + - - Empty Vector
- - + + pcDNA3-HA-ERK
- + - + H2O2

Figure 11

Fig 11: ERK mediated PI3K and Akt dephosphorylation involved in XIAP ubiquitination and degradation (a) K562 (R) cells were preincubated with PD98059 and then treated with 30μM H_2O_2 for 14 hrs. Whole cell lysates were subjected to co-immunoprecipitation with XIAP antibody and then western blot analysis were performed with anti-Akt antibody. The lower panel shows the whole cell lysates and upper panel, the immunoprecipitates.(b) K562(R) cells were treated with 30 μM H_2O_2 for 14 hours. Then whole cell lysate were subjected to western blot analysis for checking PI3K, pPI3K, Akt, pAkt. (c) K562(R) cells were treated with increasing doses of wortmannin for18 hours and then whole cell lysates were subjected to western blot analysis for checking FLIP and XIAP expression. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (d) K562(R) cells were preincubated with 10 μM wortmannin and then treated with 30 μM H_2O_2 for 18 hours. Cell lysate were subjected to western blot. XIAP and FLIP protein levels were checked.(e) K562(R) cells treated with 30 μM H_2O_2 either in presence or absence of 30 μM PD and western blot analysis was done for checking pPI3K and pAKT. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (f) K562(R) cells were transfected with either pcDNA3- HA-ERK2 or empty vector and then treated with 30μM H_2O_2 for 18 hrs. Western blot analysis was done for checking pPI3K, pAkt and ERK level. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

ITCH is responsible for JNK mediated FLIP ubiquitination and degradation:

Data indicate that H_2O_2 activated JNK, which downregulated FLIP in K562(R) cells. FLIP ubiquitination is possible by binding to ubiquitin ligase, ITCH [31,32].Cells were treated with H_2O_2 for 10hrs, cell-lysate was coimmunoprecipitated with FLIP-antibody, western blot with ITCH antibody. Result indicated that ITCH binds with FLIP in presence of H_2O_2 (Fig12a). H_2O_2 significantly increase ITCH at protein level (FIG12b). Real time PCR data reveal that ITCH expression was increased at mRNA level in presence of ROS (Fig12c). Since FLIP is downregulated by activated JNK, cells were treated with H_2O_2 alone in combination with SP600125. ITCH was coimmunoprecipitated with FLIP antibody. Coimmunoprecipitation data indicated that amount of ITCH decreased in presence of JNK inhibitor SP600125 than H_2O_2 alone (Fig12d). We checked the effect of JNK on ITCH, cells were treated with either H_2O_2 alone or in presence of SP600125. Data revealed that SP600125 reversed H_2O_2 mediated increase of ITCH (Fig12e). ERK didn't play any role in ITCH upregulation. To confirm this, cells were treated with H_2O_2 either alone or in combination with PD98059. Result showed that ITCH did not change in presence of ERK inhibitor PD than H_2O_2 alone (Fig12f). So, H_2O_2 increases ITCH at mRNA level by activation of JNK then ITCH associates with FLIP and degrades FLIP.

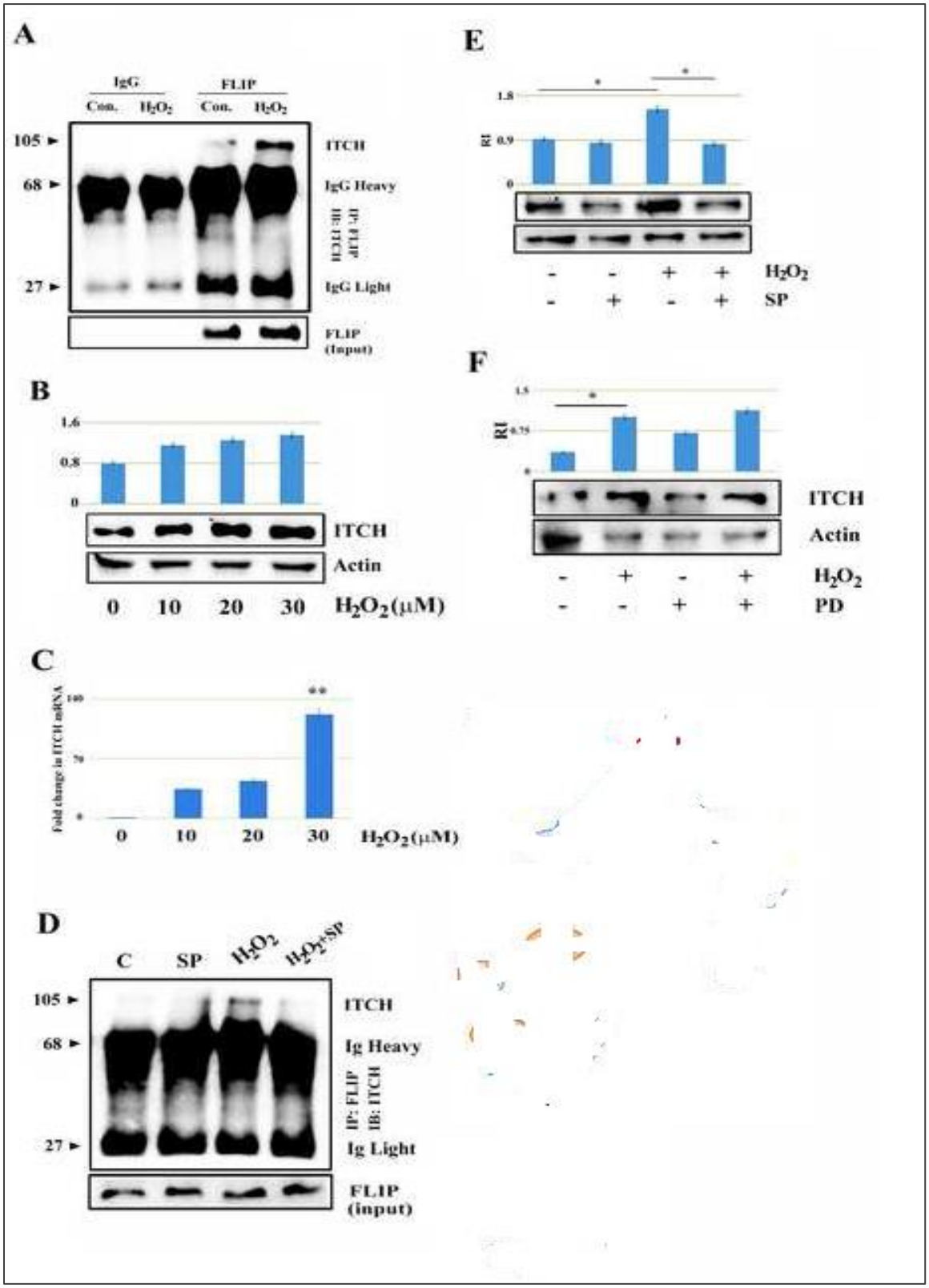

A
IgG
FLIP
Con.
H2O2
Con.
H2O2
105
68
27
ITCH
IgG Heavy
IP: FLIP
IB: ITCH
IgG Light
FLIP
(Input)
B
1.6
0.8
0
ITCH
Actin
0
10
20
30
H2O2(μM)
C
Fold change in ITCH mRNA
100
50
0
**
0
10
20
30
H2O2(μM)
D
C
SP
H2O2
H2O2+SP
105
68
27
ITCH
Ig Heavy
IP: FLIP
IB: ITCH
Ig Light
FLIP
(input)
E
1.8
0.9
0
RI
*
*
H2O2
SP
F
1.5
0.75
0
RI
*
ITCH
Actin
H2O2
PD

Figure 12

Fig12: FLIP degradation is mediated by increased expression of ITCH (a) K562 (R) cells were treated with 30μM H_2O_2 for 11 hours and the whole cell lysates were subjected to co-Immunoprecipitation with FLIP antibody and control IgG and then western blot analysis were performed with anti-ITCH antibody. The lower panel shows the whole cell lysates and upper panel, the immunoprecipitates. (b) Cells were treated with increasing concentration of H_2O_2 for 11 hrs and whole cells lysate were subjected with western blot analysis for checking ITCH at protein level. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (c) K562(R) cells were treated with increasing doses of H_2O_2 and real time PCR was performed for checking ITCH expression at protein level. Data was normalized with actin as housekeeping gene and presented graphically as fold change relative to control. Data indicates mean ± SD of three independent experiments. **$p < 0.01$ (d) K562 (R) cells were treated with either 30μM H_2O_2 alone or in combination of SP600125 for 11 hours and the whole cell lysates were subjected to co-Immunoprecipitation with FLIP antibody and then western blot analysis were performed with anti-ITCH antibody. The lower panel shows the whole cell lysates and upper panel, the immunoprecipitates.(e) K562 (R) cells were treated with SP600125 alone or in combination of H_2O_2. Western blot analysis was done for checking the level of ITCH at protein level. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (f) K562 (R) cells were treated with PD98059 alone or in combination of H_2O_2. Western blot analysis was done for checking the level of ITCH at protein level. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

From objective II, it is confirmed that ROS downregulates XIAP, FLIP by ubiquitin proteosomal pathway. ROS-activated ERK decreases Akt phosphorylation that inhibits binding of Akt to XIAP. ROS increases ITCH at protein level by activation of JNK. ITCH (E3 ubiquitin ligase) associates with FLIP and degrades it by ubiquitin-proteasome pathway.

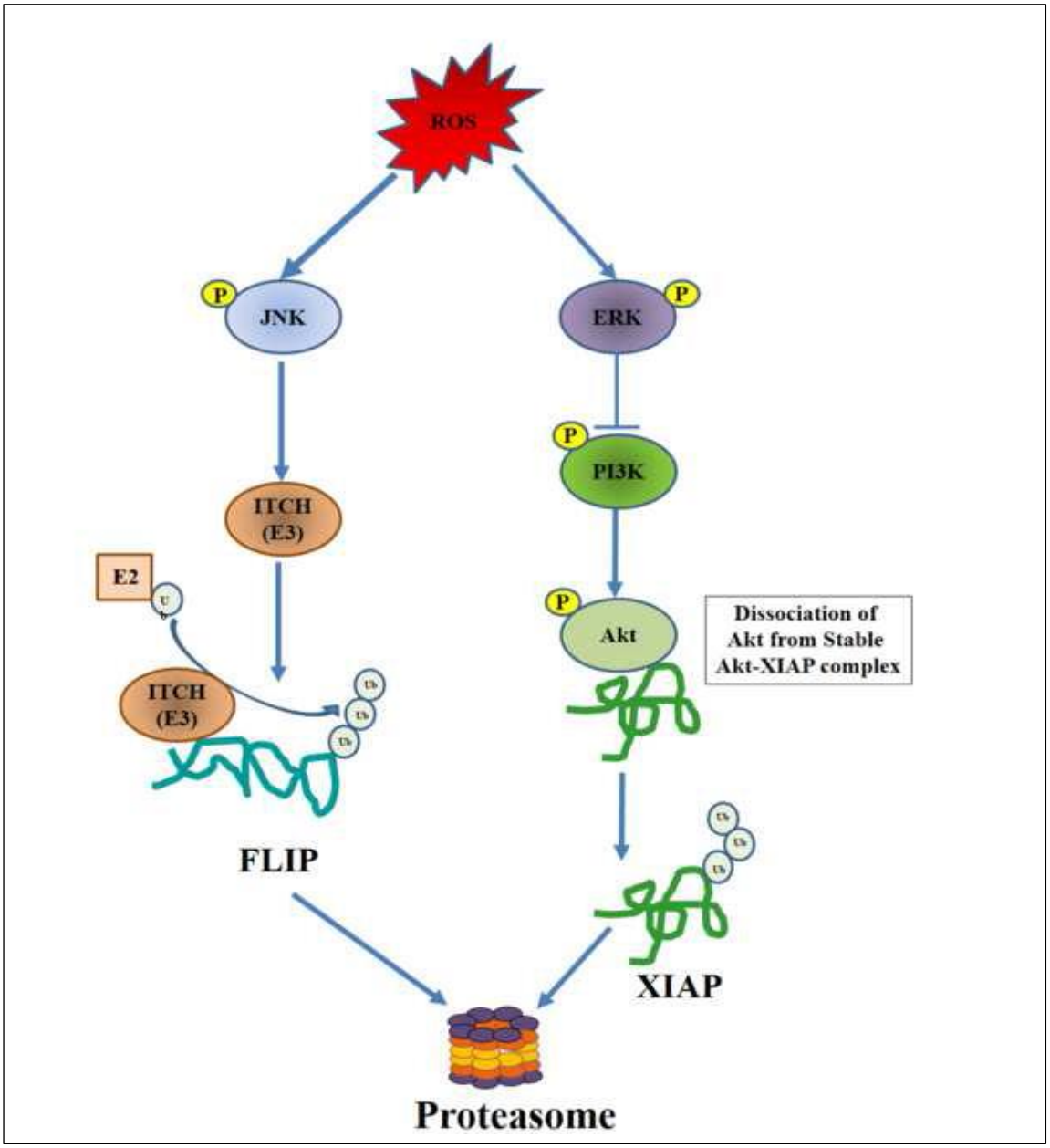


Figure 13

Figure 13 : ROS mediated XIAP,FLIP proteosomal degradation via MAPK kinase pathway in imatinib resistant K562 cells. ROS decreases anti-apoptotic protein XIAP and FLIP by activation of ubiquitin proteasomal pathway. ROS-activated ERK subsequently decreases Akt phosphorylation which inhibits the binding of Akt to the XIAP and increases its ubiquitin mediated degradation. On the other hand ROS increases ITCH at protein level by activation of JNK. ITCH, being an E3 ubiquitin ligase, associates with FLIP and degrades it by ubiquitin-proteasome pathway

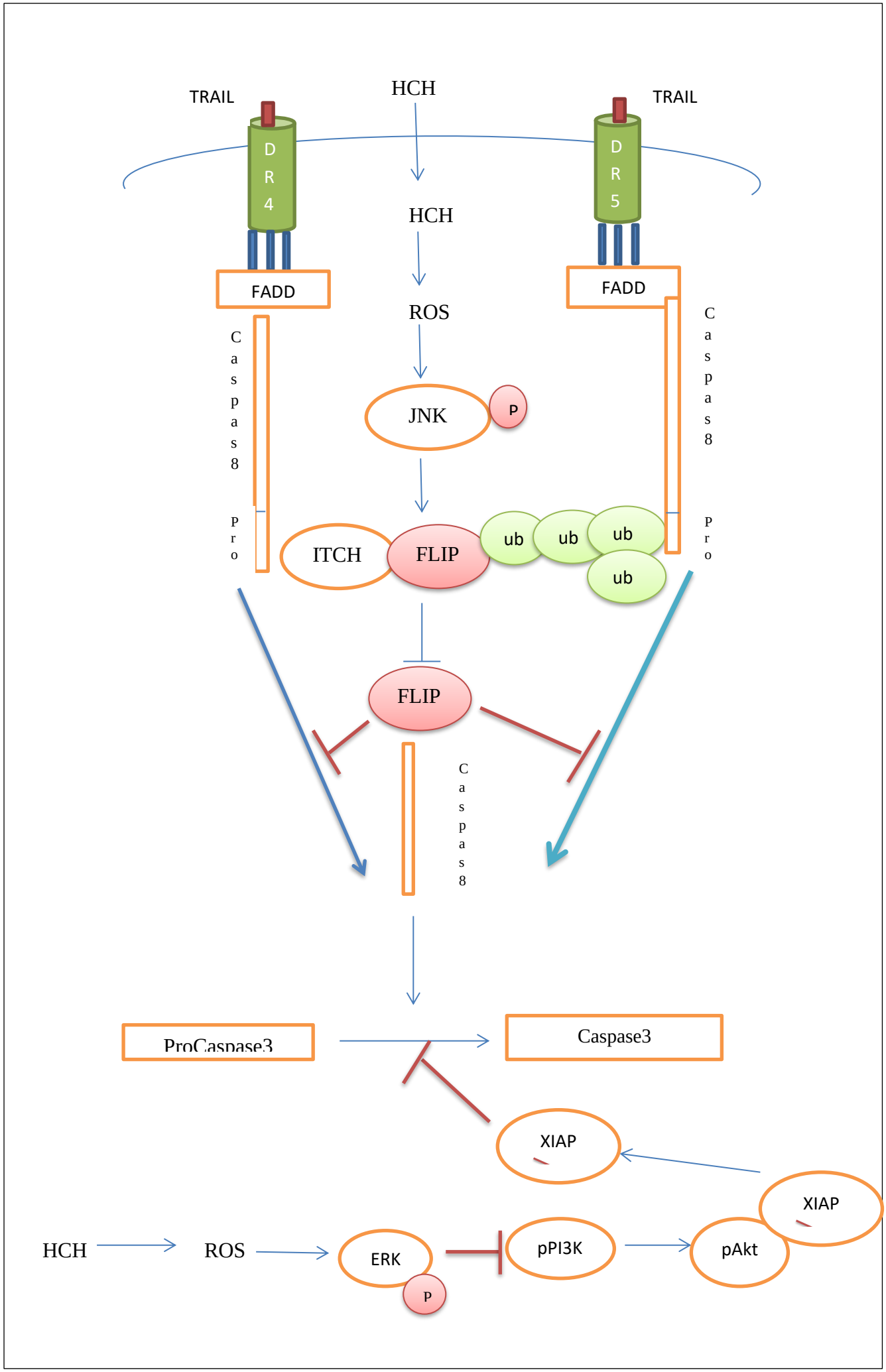

TRAIL
HCH
TRAIL
DR4
DR5
HCH
FADD
FADD
ROS
Caspas8
Pro
JNK
P
Caspas8
Pro
ITCH
FLIP
ub
ub
ub
ub
FLIP
Caspas8
ProCaspase3
Caspase3
XIAP
XIAP
HCH
ROS
ERK
P
pPI3K
pAkt

Fig: Hydroxychavicol downregulates anti apoptotic protein XIAP,FLIP via ROS generation. MAPK pathway involved in this downregulation in imatinib resistant K562 cells. HCH induces ROS inside cells specially H_2O_2. . ROS decreases anti-apoptotic protein XIAP and FLIP by activation of ubiquitin proteasomal pathway. ROS-activated ERK subsequently decreases Akt phosphorylation which inhibits the binding of Akt to the XIAP and increases its ubiquitin mediated degradation. On the other hand ROS increases ITCH at protein level by activation of JNK. ITCH, being an E3 ubiquitin ligase, associates with FLIP and degrades it by ubiquitin-proteasome pathway. In this way, ROS downregulates XIAP and FLIP . HCH sensitize imatinib-resistant CML cells to TRAIL-induced apoptosis through downregulation of XIAP and FLIP via ROS generation.

Objective III: **To check the key findings in imatinib resistant cells from objective I and objective II in imatinib sensitive cells.**

The followings are the key findings from object I and II.

1. Hydroxychavicol downregulates XIAP, FLIP via ROS generation.
2. H_2O_2 downregulates XIAP, FLIP by ubiquitin proteosomal pathway.
3. H_2O_2 mediated ERK and JNK activation cause XIAP and FLIP downregulation respectively.
4. ROS activated ERK decreases PI3K/Akt phosphorylation that inhibit binding of Akt to XIAP. So, XIAP autoubiquitination is possible.
5. ROS increases ITCH expression at protein level by activation of JNK. ITCH associates with FLIP and degrades it by ubiquitin proteosome pathway.

Next, the above key results from imatinib resistant CML cell line K562 have now been checked in Imatinib-sensitive CML cell lines K562.

H_2O_2 downregulates XIAP, FLIP in Imatinib sensitive cells by lysosomal degradation pathway

To check the effect of Hydroxychavicol on XIAP, FLIP downregulation in imatinib sensitive cells, cells are treated with different doses of Hydroxychavicol. Result show that XIAP, FLIP downregulation were increased with increasing concentration of Hydroxychavicol (Fig14a). Hydroxychavicol downregulates XIAP, FLIP in ROS dependant manner, specifically H_2O_2 dependant manner. To check effect of ROS on XIAP, FLIP downregulation, both cells lines were treated with 30 μM H_2O_2. Result indicates that H_2O_2 decreases XIAP and FLIP at protein level (Fig14b). We observed that XIAP and FLIP both level are decreased in imatinib resistant cells. When cells were pretreated with NAC followed by H_2O_2, NAC significantly reversed XIAP, FLIP downregulation (Fig14c). To understand the molecular mechanism behind FLIP, XIAP downregulation, K562(S) were treated with MG132 before incubation with H_2O_2, result showed that MG132 didn't reverse H_2O_2 mediated XIAP and FLIP downregulation (Fig14d). So, H_2O_2 mediated downregulation of FLIP, XIAP is not proteosomal mediated in k562 (S) cells. In contrast, H_2O_2 mediated XIAP, FLIP downregulation is occurred via proteosomal dependant in K562 (R) cells.

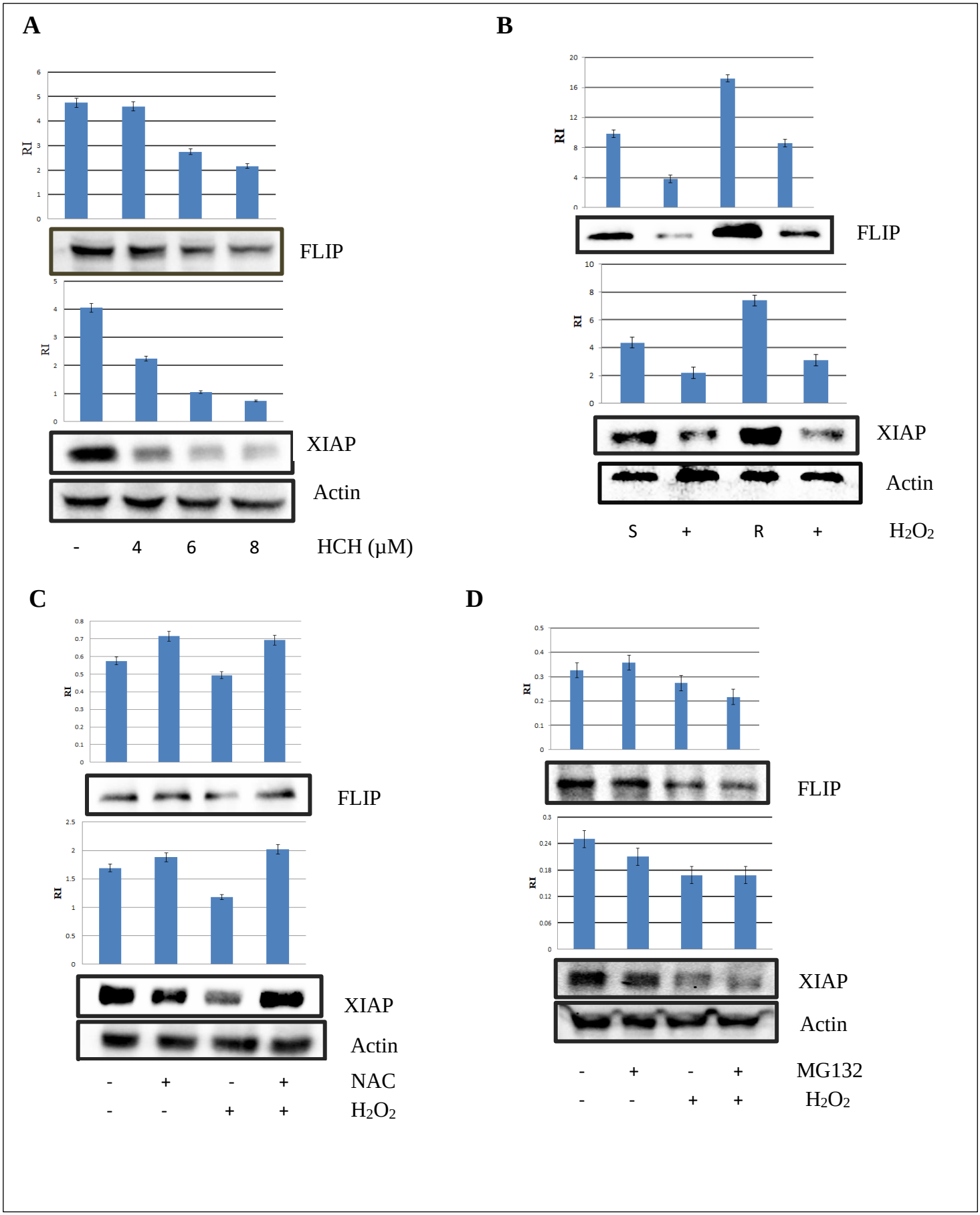

A
RI
FLIP
XIAP
Actin
- 4 6 8
HCH (μM)
B
RI
FLIP
XIAP
Actin
S + R +
H2O2
C
RI
FLIP
XIAP
Actin
- + - +
NAC
- - + +
H2O2
D
RI
FLIP
XIAP
Actin
- + - +
MG132
- - + +
H2O2

Figure 14

Fig14: H_2O_2 downregulates XIAP, FLIP in Imatinib sensitive cells via lysosomal degradation pathway. a) K562(S) cells were treated with different doses of Hydroxychavicol for 18 hrs. Western blot analysis was done for checking the level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis. b) Imatinib sensitive K562 cells and imatinib resistant K562 cells were treated with H_2O_2 for 18 hrs. Western blot analysis was done for checking the level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis**.** C) K562 cells were pre-incubated with 1mM NAC for 1 hr and then treated with 30μM H_2O_2 for overnight. Whole cells lysate were subjected to western blot analysis for checking protein level of XIAP, FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.d) Imatinib sensitive K562 cells were treated with MG132 followed by treated with H_2O_2 for 18 hrs. Western blot analysis was done for checking the level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

ROS is involved in activation of JNK but downregulation of ERK activity in imatinib sensitive cells:

We have checked whether ROS can modulate MAPK pathway. So, K562 (S) cells were treated with different doses of H_2O_2. Result showed that H_2O_2 increase JNK phosphorylation but decrease ERK phosphorylation in K562(S) cells. (Fig 15a).In contrast, H_2O_2 increases phosphorylation of JNK and ERK in K562(R).

A

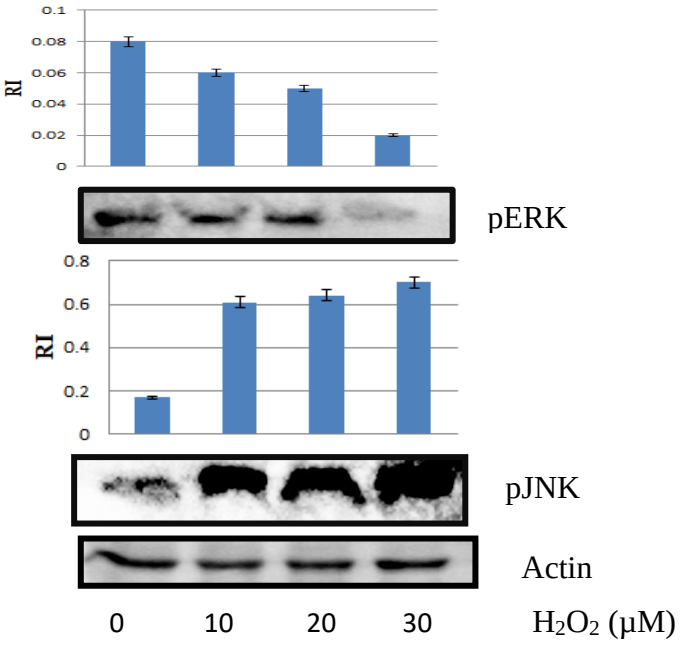


Figure 15

Fig15 : ROS is involved in activation of JNK but downregulation of ERK activity a) Imatinib sensitive K562 cells were treated with H_2O_2 for for 14 hrs. Western blot analysis was done for checking the level of phosphorylation level of JNK and ERK. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

Activated JNK involved in XIAP and FLIP downregulation both in K562(S) cells:

We checked whether JNK pathway was involved in this downregulation. Cells were incubated with SP600125 then treated with H_2O_2. Result indicated that SP reversed H_2O_2 mediated FLIP, XIAP down-regulation after 18 hrs (Fig16a). So, ROS mediated activated JNK is involves in XIAP and FLIP downregulation.

A

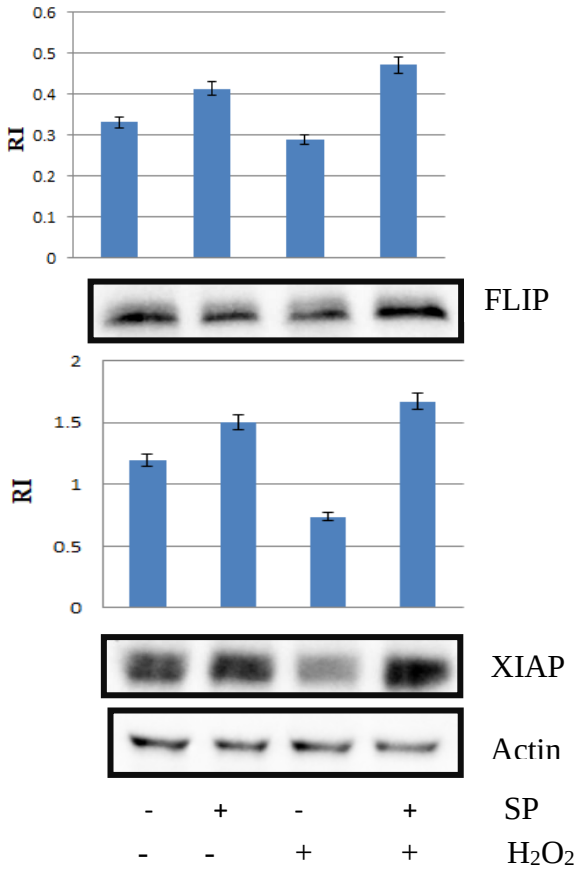


Figure 16

Fig16: Activated JNK is involved in XIAP and FLIP downregulation both in Imatinib-sensitive K562 cells a) Imatinib sensitive K562 cells were treated with SP followed by treated with H_2O_2 for 18 hrs. Western blot analysis was done for checking the level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

Activated ERK involved in XIAP and FLIP upregulation in K562 (S) cells:

Cells were incubated with PD98059, then treated with H_2O_2 for 18 hrs. Result indicated that PD increases H_2O_2 mediated FLIP, XIAP downregulation (fig 17a). It is concluded that in absence of ROS, ERK upregulates XIAP and FLIP.

A

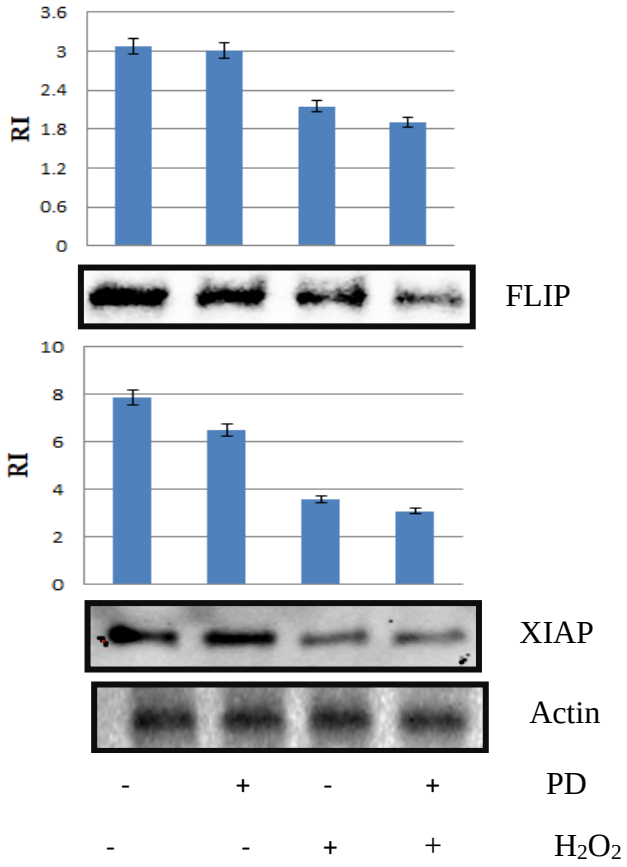


Figure 17

Fig17: Activated ERK involved in XIAP and FLIP upregulation in Imatinib-sensitive K562 cells: **a)** Imatinib sensitive K562 cells were treated with PD followed by treated with H_2O_2 for 18 hrs. Western blot analysis was done for checking the level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

H_2O_2 do not increase ITCH expression in K562 (S) cells:

To check the effect of ROS on ITCH, K562(S) cells were treated with different doses of H_2O_2 for 10 hours and result showed that ITCH is little bit decreased at protein level at 30 µM H_2O_2 (Fig18a). So, ITCH has no role in XIAP, FLIP degradation in K562 (S) cells. In-contrast, H_2O_2 increase expression of ITCH in K562(R) cell, play role in FLIP degradation.

A

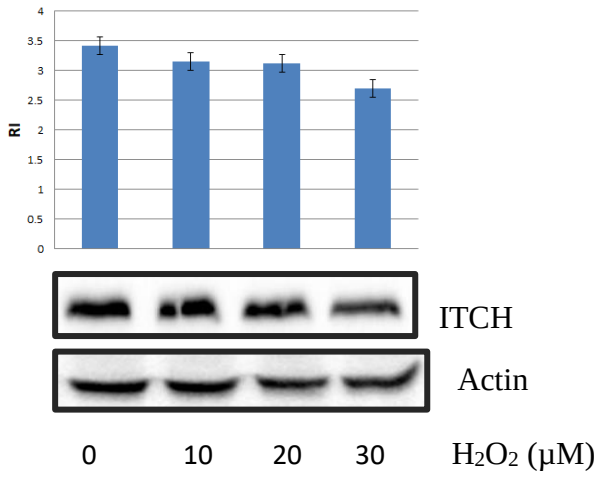


Figure 18

Fig18**:** H_2O_2 do not increase ITCH expression in Imatinib sensitive K562 cells: a) Immunobloting of ITCH was performed using whole cell lysate of K562 (S) cells under indicated concentration of H_2O_2 treatment for 11 hours. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

H_2O_2 mediated ERK dephosphorylation causes PI3K, Akt dephosphorylation, thereby enhances XIAP, FLIP downregulation in K562 (S) cell

Cells were treated with different doses of H_2O_2 for 14 hrs. Data indicated that H_2O_2 dose dependently decreased both PI3K and Akt phosphorylation level whereas Akt remains unaltered at protein level (Fig19a). To check the role of PI3K and Akt in XIAP, FLIP downregulation, cells were treated with PI3K inhibitor Wortmannin and then H_2O_2 was added, Result showed that XIAP, FLIP level decreased further than H_2O_2 alone (Fig19b). So, it is confirmed that PI3K and Akt played a crucial role in XIAP,FLIP upregulation in K562(S) cells. In contrast, PI3K, Akt played role in XIAP upregulation with minimal effect on FLIP in K562(R) cells. To check role of PI3K, Akt in XIAP downregulation, cells were treated with Wortmanin for 18 hrs. Result indicated that Wortmanin dose dependently

decreased XIAP and FLIP level (Fig19c). To assess the role of ERK in PI3K and Akt phosphorylation, K562(S) cells were treated with PD98059 then treated with H_2O_2 for 14 hours. Result indicated that PD enhances H_2O_2-mediated dephosphorylation of PI3K and Akt (Fig19d). So, In H_2O_2 treated K562(S) cells, H_2O_2 inactivate ERK, thereby Pi3k, Akt is not phosphorylated, So, XIAP,FLIP degradation is occurred.

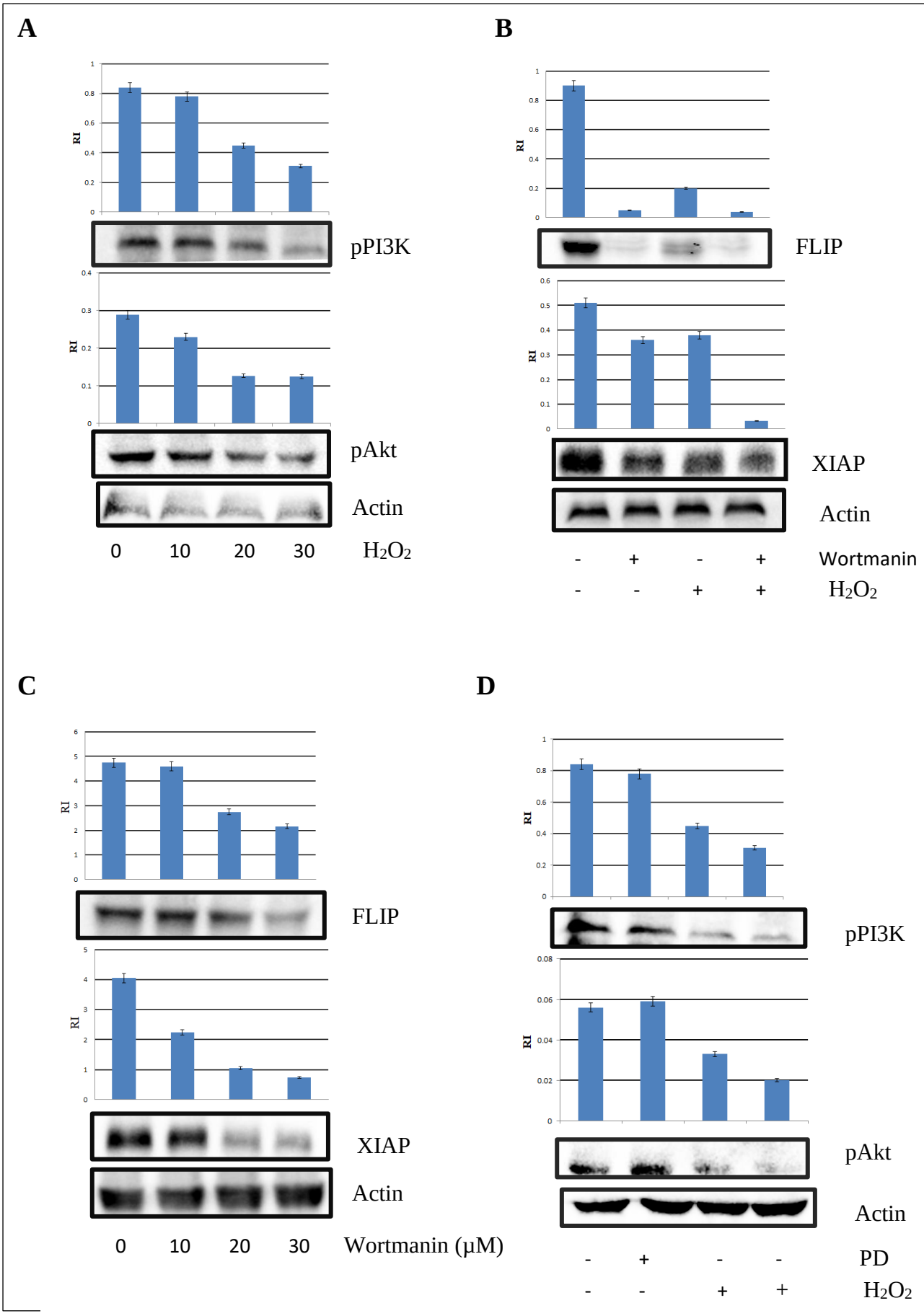

A
RI
pPI3K
pAkt
Actin
0 10 20 30 H2O2
B
RI
FLIP
XIAP
Actin
- + - + Wortmanin
- - + + H2O2
C
RI
FLIP
XIAP
Actin
0 10 20 30 Wortmanin (μM)
D
RI
pPI3K
pAkt
Actin
- + - - PD
- - + + H2O2

Figure 19

Fig19: H_2O_2 mediated ERK dephosphorylation causes PI3K, Akt dephosphorylation, thereby enhance XIAP, FLIP downregulation in imatinib sensitive K562 cell a) K562(S) cells were treated with different doses of H_2O_2 for 14 hours and then whole cell lysates were subjected to immune-blot analysis with pPI3K, pAkt antibodies. Data were presented graphically as ratio to actin after densitometry analysis. b) K562(S) cells were incubated with or without 10 μM wortmannin and 30 μM H_2O_2 for 18 hours, then cell lysates were subjected to western blot and XIAP and FLIP protein levels were checked. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis. c) K562(S) cells were treated with increasing doses of Wortmannin for18 hours and then whole cell lysates were subjected to western blot analysis for checking FLIP and XIAP expression. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis. D) K562(R) cells treated with 30 μM H_2O_2 either in presence or absence of 30 μM PD and the levels of pPI3K and pAKT was analysed by Immunoblot. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis.

Our data suggests that ROS decreases XIAP and FLIP in K562(S) cells. In contrast, ROS degrades XIAP and FLIP by proteosomal degradation pathway in K562(R) cells. ROS inactivates ERK subsequently decreases Akt phosphorylation thereby degradation of FLIP, XIAP are occurred in K562(S) cells. In contrast, ERK mediated dephosphorylation of PI3K and Akt plays a critical role in XIAP ubiquitination and degradation in presence of H2O2 in K562(R) cells. ROS activates JNK by phosphorylation thereby degradation of XIAP, FLIP is possible. ITCH is not involved in ROS mediated XIAP.FLIP downregulation. Because ITCH expression is not increased in presence of ROS in K562(S) cells. In contrast, in K562 (R), ROS increases ITCH at protein level by activation of JNK. ITCH associates with FLIP

and degrades it. In this way ROS downregulates FLIP and XIAP in K562(S) and K562(R) cells.

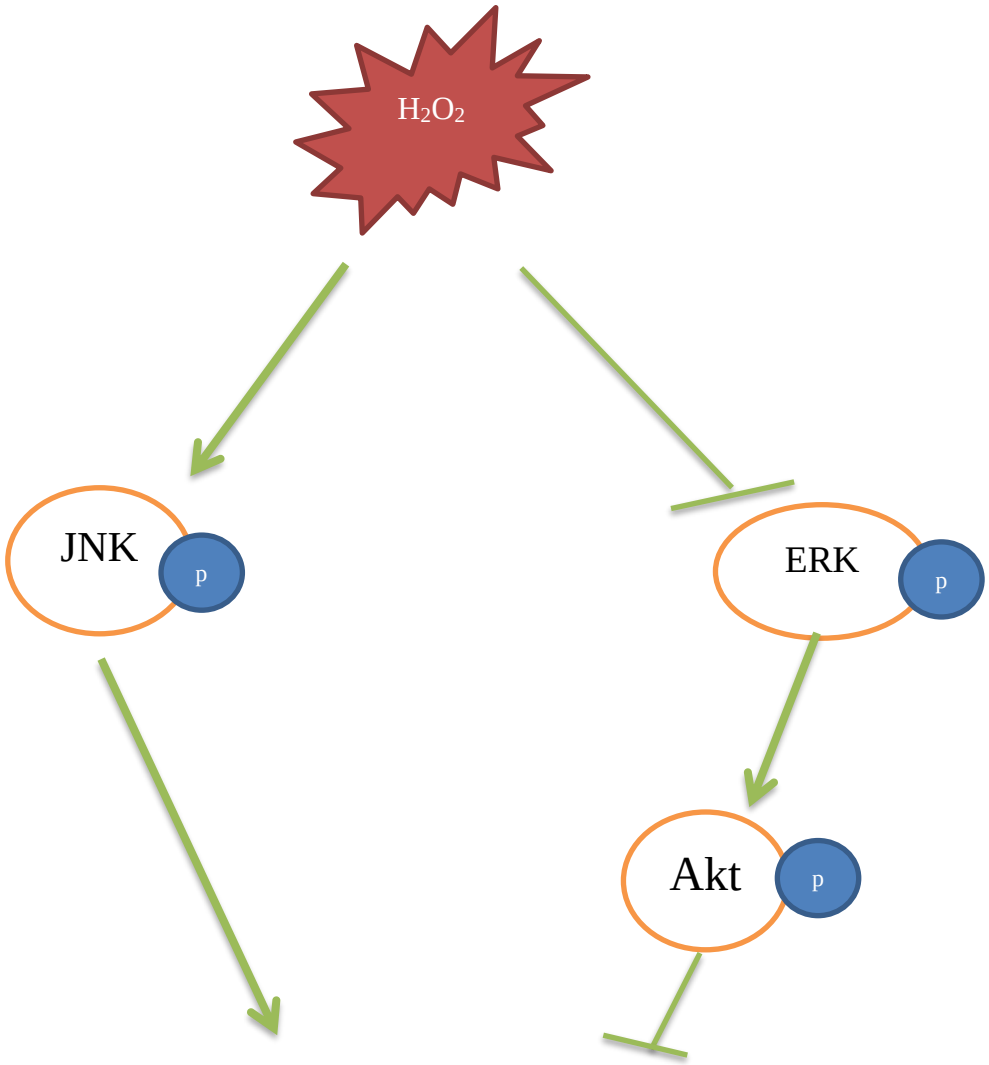


Fig: ROS mediated XIAP , FLIP degradation via MAPK kinase pathway in imatinib sensitive K562 cells

Discussion: K562 (R) cells show resistant to TRAIL mediated apoptosis. Since in a previous study Hydroxychavicol has been shown to kill K562(S) cells, we tried to sensitize K562(R) to TRAIL by using Hydroxychavicol. Combinatory treatment of HCH ,TRAIL induced K562(R) cells to TRAIL mediated apoptosis. HCH decreases XIAP, FLIP at protein level.

The down-regulation of XIAP and FLIP are responsible for TRAIL-mediated apoptosis because knocking down XIAP and FLIP by siRNA also similarly increased TRAIL-induced apoptosis. In previous study, HCH showed to generate ROS (33). Here in this study, its was found that ROS was induced by HCH. This ROS played role in the enhancement of apoptosis by TRAIL. Exogenous ROS that was applied to K562(R) cells also decreased XIAP and FLIP levels which confirms ROS as a player in the downregulation of XIAP and FLIP. So, from objective I, it is confirmed that IAPs can targeted by HCH via H_2O_2 dependant manner to sensitize K562(R) cells to TRAIL mediates apoptosis.

In objective II, we examined the signalling mechanism that is involved in H_2O_2 dependant XIAP, FLIP downregulation. ROS decreases FLIP, XIAP by activation of ubiquitin proteasomal pathway. ROS-activated ERK subsequently decreases Akt phosphorylation which inhibits binding of Akt to the XIAP, increases its ubiquitin mediated degradation. ROS increases ITCH at protein level by activation of JNK. ITCH, an E3 ubiquitin ligase, associates with FLIP cause degradation of FLIP by ubiquitin-proteasome pathway. So ROS degrades XIAP,FLIP.

We have found some key finding in K562(S) cells from objective I ,II in K562(R) cells . ROS degrades XIAP,FLIP in imatinib sensitive K562 cells as similar to K562 (R). But ROS mediated XIAP,FLIP degradation pathways are different between K562(S) and K562(R) cells. In K562(S) cells, ROS inactivates ERK pathway, thereby Akt dephosphorylation is occurred, XIAP,FLIP degradation is possible. Activated ERK is involved in XIAP,FLIP upregulation. But in K562(R) cells, ROS activated ERK involved XIAP degradation. In K562(S) cells, ROS activated JNK is involved in XIAP, FLIP degradation. In K562(R) cells, activated JNK is involved in degradation of FLIP by ubiquitination proteosomal mediated pathway.

Therapeutic implications of this study:

XIAP and FLIP both are antiapoptotic proteins. So, downregulation of these two proteins lead to apoptosis in K562 cells. In our study it has been shown that downregulation of these two proteins leads to TRAIL induced apoptosis in imatinib resistant K562 cells. In our study we also have targeted PI3K/Akt for anti leukemic treatment in imatinib resistant K562 cells. This might lead to the alternative approach for the treatment of Imatinib resistant leukemic patients who are otherwise also resistant to TRAIL.

Future Direction: We studied that ROS regulates MAPK kinase signaling pathway in Imatinib sensitive and resistant K562 cells. We studied that the mechanisms by which ROS mediated MAPK kinase signaling pathway regulate antiapoptotic proteins FLIP, XIAP expression. In future, we will find out several signaling pathways other than MAPK pathway that were regulated by ROS. Then we will study the mechanisms by which these ROS mediated signalling pathways regulates several proapoptotic proteins and antiapoptotic proteins.

Reference

1. Savona M, Talpaz M. Getting to the stem of chronic myeloid leukaemia. Nat Rev Cancer 2008; 8:341–350.

2. Aggarwal BB. Signalling pathways of the TNF superfamily: a double edged sword. Nat Rev Immunol 2003; 3:745–756.

3. S. Fulda, A.M. Gorman, O. Hori, A. Samali, Cellular stress responses: Cell survival and cell death, Int. J. Cell Biol. (2010)

4. J.M. Vicencio, L. Galluzzi, N. Tajeddine, C. Ortiz, A. Criollo, E. Tasdemir, E. Morselli, A. Ben Younes, M.C. Maiuri, S. Lavandero, G. Kroemer, Senescence, apoptosis or autophagy? When a damaged cell must decide its path - A mini-review, Gerontology. 54 (2008) 92–99.

5. K. Labbé, M. Saleh, Cell death in the host response to infection, Cell Death Differ. 15 (2008) 1339–1349.

6. C. Chen, T.S. Liu, S.C. Zhao, W.Z. Yang, Z.P. Chen, Y. Yan, XIAP impairs mitochondrial function during apoptosis by regulating the Bcl-2 family in renal cell carcinoma., Exp. Ther. Med. 15 (2018) 4587–4593.

7. L. Flanagan, J. Kehoe, J. Fay, O. Bacon, A.U. Lindner, E.W. Kay, J. Deasy, D.A. McNamara, J.H.M. Prehn, High levels of X-linked Inhibitor-of-Apoptosis Protein (XIAP) are indicative of radio chemotherapy resistance in rectal cancer., Radiat. Oncol. 10 (2015) 131.

8. L. Humphreys, M. Espona-Fiedler, D.B. Longley, FLIP as a therapeutic target in cancer., FEBS J. 285 (2018) 4104–4123.

9. S. Horn, M.A. Hughes, R. Schilling, C. Sticht, T. Tenev, M. Ploesser, P. Meier, M.R. Sprick, M. MacFarlane, M. Leverkus, Caspase-10 Negatively Regulates Caspase-8-Mediated Cell Death, Switching the Response to CD95L in Favor of NF-κB Activation and Cell Survival., Cell Rep. 19 (2017) 785–797.

10. G. Valente, F. Manfroi, C. Peracchio, G. Nicotra, R. Castino, G. Nicosia, S. Kerim, C. Isidoro, cFLIP expression correlates with tumour progression and patient outcome in non-Hodgkin lymphomas of low grade of malignancy., Br. J. Haematol. 132 (2006) 560–70.

11. M.G. Hinds, R.S. Norton, D.L. Vaux, C.L. Day, Solution structure of a baculoviral inhibitor of apoptosis (IAP) repeat, Nat. Struct. Biol. 6 (1999) 648–651

12. C. Sun, M. Cai, R.P. Meadows, N. Xu, A.H. Gunasekera, J. Herrmann, J.C. Wu, S.W. Fesik, NMR structure and mutagenesis of the third Bir domain of the inhibitor of apoptosis protein XIAP, J. Biol. Chem. 275 (2000) 33777–33781.

13. Y. Suzuki, Y. Nakabayashi, R. Takahashi, Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death., Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 8662–7.

14. Y. Yang, S. Fang, J.P. Jensen, A.M. Weissman, J.D. Ashwell, Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli, Science (80-.). 288 (2000) 874–877.

15. J. Silke, T. Kratina, D. Chu, P.G. Ekert, C.L. Day, M. Pakusch, D.C.S. Huang, D.L. Vaux, Determination of cell survival by RING-mediated regulation of inhibitor of apoptosis (IAP) protein abundance., Proc.Natl. Acad. Sci. U. S. A. 102 (2005) 16182–7.

16. H.H. Cheung, S. Plenchette, C.J. Kern, D.J. Mahoney, R.G. Korneluk, The RING domain of cIAP1 mediates the degradation of RING-bearing inhibitor of apoptosis proteins by distinct pathways, Mol. Biol. Cell. 19 (2008) 2729–2740.

17. M.A. Hughes, I.R. Powley, R. Jukes-Jones, S. Horn, M. Feoktistova, L. Fairall, J.W.R. Schwabe, M. Leverkus, K. Cain, M. MacFarlane, Co-operative and Hierarchical Binding of c-FLIP and Caspase-8: A Unified Model Defines How c-FLIP Isoforms Differentially Control Cell Fate.,

18. K. Schleich, J.H. Buchbinder, S. Pietkiewicz, T. Kähne, U. Warnken, S. Öztürk, M. Schnölzer, M. Naumann, P.H. Krammer, I.N. Lavrik, Molecular architecture of the

DED chains at the DISC: regulation of procaspase-8 activation by short DED proteins c-FLIP and procaspase-8 prodomain., Cell Death Differ. 23 (2016) 681–94.

19. C. Haag, D. Stadel, S. Zhou, M.G. Bachem, P. Möller, K.M. Debatin, S. Fulda, Identification of c-FLIPL and c-FLIPS as critical regulators of death receptor-induced apoptosis in pancreatic cancer cells, Gut. 60 (2011) 225–237.

20. T.W. Day, S. Huang, A.R. Safa, c-FLIP knockdown induces ligand-independent DR5-, FADD-, caspase-8-, and caspase-9-dependent apoptosis in breast cancer cells., Biochem. Pharmacol. 76 (2008) 1694–704.

21. X. Zhang, L. Zhang, H. Yang, X. Huang, H. Otu, T.A. Libermann, W.C. DeWolf, R. Khosravi-Far, A.F. Olumi, c-Fos as a proapoptotic agent in TRAIL-induced apoptosis in prostate cancer cells, Cancer. 67(2007) 9425–9434

22. Aggarwal BB. Signalling pathways of the TNF superfamily: a doubleedged sword. Nat Rev Immunol 2003; 3:745–756.

23. Aggarwal BB, Bhardwaj U, Takada Y. Regulation of TRAIL-induced apoptosis by ectopic expression of antiapoptotic factors. Vitam Horm 2004; 67:453–483.

24. Biswas N, Mahato SK, Chowdhury AA, Chaudhuri J, Manna A, Vinayagam J, et al. ICB3E induces iNOS expression by ROS-dependent JNK and ERK activation for apoptosis of leukemic cells. Apoptosis 2012; 17:612–626.

25. Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: cell survival and cell death. Int J Cell Biol 2010; 2010:214074.

26. Sakamoto S, Kyprianou N. Targeting anoikis resistance in prostate cancer metastasis. Mol Aspects Med 2010; 31:205–214.

27. Silke J, Meier P. Inhibitor of apoptosis (IAP) proteins-modulators of cell death and inflammation. Cold Spring Harb Perspect Biol 2013; 5:a008730.

28. Lopes RB, Gangeswaran R, McNeish IA, Wang Y, Lemoine NR. Expression of the IAP protein family is dysregulated in pancreatic cancer cells and is important for resistance to chemotherapy. Int J Cancer 2007; 120:2344–2352.

29. Hinz S, Trauzold A, Boenicke L, Sandberg C, Beckmann S, Bayer E, et al. Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAILreceptor mediated apoptosis. Oncogene 2000; 19:5477–5486

30. Fulda S, Küfer MU, Meyer E, van Valen F, Dockhorn-Dworniczak B, Debatin KM. Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer. Oncogene 2001; 20:5865–5877.

31. Eggert A, Grotzer MA, Zuzak TJ, Wiewrodt BR, Ho R, Ikegaki N, Brodeur GM. Resistance to tumornecrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with aloss of caspase-8 expression. Cancer Res 2001; 61:1314–1319.

32. Hao XS, Hao JH, Liu FT, Newland AC, Jia L. Potential mechanisms of leukemia cell resistance to TRAIL-induced apopotosis. Apoptosis 2003; 8:601–607.

33. Ashkenazi A, Dixit VM. Apoptosis control by death and decoy receptors. Curr Opin Cell Biol 1999; 11:255–260.

34. MacFarlane M. TRAIL-induced signalling and apoptosis. Toxicol Lett 2003; 139:89–97.

35. Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: cell survival and cell death. Int J Cell Biol 2010; 2010:214074.

36. Sakamoto S, Kyprianou N. Targeting anoikis resistance in prostate cancer metastasis. Mol Aspects Med 2010; 31:205–214.

37. Geiger TR, Peeper DS. Metastasis mechanisms. Biochim Biophys Act 2009; 1796:293–308.

38. Fulda S. Tumor resistance to apoptosis. Int J Cancer 2009; 124:511–515

39. Westhoff MA, Fulda S. Adhesion-mediated apoptosis resistance in cancer. Drug Resist Updat 2009; 12:127–136

Plagiarism Certificate

Curiginal

Document Information

Analyzed document	5000 words only.docx (D121133076)
Submitted	2021-12-05T07:55:00.0000000
Submitted by	Nabendu Biswas
Submitter email	nabendu.dbs@presiuniv.ac.in
Similarity	1%
Analysis address	nabendu.dbs.presi@analysis.urkund.com

Sources included in the report

W	URL: https://www.x-mol.com/paper/1363968201831256064 Fetched: 2021-12-05T07:57:00.0000000	1
J	URL: b971d91f-e7bc-47a1-9d5a-9b1e76819851 Fetched: 2019-03-18T18:40:16.4970000	1

Publication paper

› Anticancer Drugs. 2019 Feb;30(2):167-178. doi: 10.1097/CAD.0000000000000710.

Hydroxychavicol sensitizes imatinib-resistant chronic myelogenous leukemia cells to TRAIL-induced apoptosis by ROS-mediated IAP downregulation

Tamalika Paul [1], Anupam Banerjee [1], S Vijaya Bhaskara Reddy [2], Sanjit K Mahato [3], Nabendu Biswas [1]

Affiliations + expand

PMID: 30418193 DOI: 10.1097/CAD.0000000000000710

Abstract

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of cytokine superfamily, induces apoptosis in a number of tumor cells through the activation of extrinsic apoptotic pathway but shows little or no cytotoxicity toward normal cells. However some tumor cells are inherently resistant to TRAIL-mediated apoptosis, which needs to be addressed to establish TRAIL as a potential chemotherapeutic drug. In this study, our aim was to manipulate TRAIL-apoptosis pathway by hydroxychavicol (HCH), a polyphenol from Piper betel leaf, for the induction of apoptosis in TRAIL resistant chronic myeloid leukemia cell. When imatinib-resistant K562 cells were treated with HCH, it made these K562 cells sensitive to TRAIL. It was observed that HCH downregulated antiapoptotic proteins XIAP and FLIP, whereas the expression of TRAIL receptors, DR4 and DR5, remains unchanged. Moreover, we observed that reactive oxygen species or ROS played a crucial role in the downregulation of FLIP and XIAP because ROS scavenger significantly reversed the decrease of XIAP, and FLIP. Ubiquitin-proteasome pathway was observed to play a crucial role in the downregulation of XIAP and FLIP, as proteasomal inhibitor MG132 significantly reversed the downregulation of XIAP and FLIP. In conclusion, this study demonstrates the combinatorial treatment of TRAIL and HCH as promising alternative therapeutic approach to treat the imatinib-resistant leukemia, which are also resistant to TRAIL.

› Free Radic Biol Med. 2021 Apr;166:265-276. doi: 10.1016/j.freeradbiomed.2021.02.024. Epub 2021 Feb 22.

H_2O_2 mediated FLIP and XIAP down-regulation involves increased ITCH expression and ERK-Akt crosstalk in imatinib resistant Chronic Myeloid Leukemia cell line K562

Tamalika Paul [1], Rajdeep Roy [1], Rupak Dey Sarkar [1], Samraj Sinha [1], Nabendu Biswas [2]

Affiliations + expand

PMID: 33631302 DOI: 10.1016/j.freeradbiomed.2021.02.024

Abstract

Regulation of anti-apoptotic protein FLICE-like inhibitory protein (FLIP) and X-linked inhibitor of apoptosis protein (XIAP) remains a crucial step in the cell fate determination and thus targeting these anti-apoptotic proteins could be a viable strategy for the treatment of cancer. However the regulation of FLIP and XIAP is not very well established till date. Here we have shown that ROS decreased XIAP and FLIP by activation of ubiquitin-proteasomal pathway in imatinib resistant K562 cells. Activation of the components of MAPK pathway, ERK and JNK, played a crucial role in XIAP and FLIP degradation because ectopic expression or knock down of ERK and JNK changed the pattern of ROS mediated down-regulation of these two proteins. We have also found that JNK and ERK differentially regulates FLIP and XIAP, respectively. Moreover, our data suggests that activated ERK decreased Akt phosphorylation and thus its binding to and stabilization of XIAP. On the other hand, JNK activation increased E3 ubiquitin ligase ITCH expression and its binding to FLIP which leads to its degradation. Thus, we have, for the first time elucidated that ROS mediated ERK-Akt crosstalk regulates XIAP. We have also shown for the first time that ROS regulates ITCH expression which controls FLIP degradation.

Keywords: ERK; FLIP; Itch; JNK; ROS; XIAP.

Copyright © 2021 Elsevier Inc. All rights reserved.

Conference Certificate

11th East Zonal Oncology CME

(under the banner of IASO & ASI, W.B.)

Organised by

SAROJ GUPTA CANCER CENTRE & RESEARCH INSTITUTE
THAKURPUKUR, KOLKATA

THEME : Clinical and Molecular Advances in Breast Cancer

On 22nd February | 2020

Certificate Presented to

Dr./ Mr./Ms. Tamalika Paul

"Is Certified in recognition of Poster Presentation."

Prof. (Dr.) Arnab Gupta
Chairman, Organizing Committee
President - IASO

Dr. Bitan Chattopadhyay
President
ASI (W.B.)

Prof. (Dr.) Biman Chakraborty
Chairman, Organizing Committee

Dr. Somsubhra Nath
Jt. Organizing Secretary

Dr. Syed Hassanujjaman
Jt. Organizing Secretary

"आत्मनिर्भरभारताय विज्ञानस्य च विज्ञानम्"
Science for Self-Reliant India and Global Welfare

INDIA INTERNATIONAL SCIENCE FESTIVAL 2020

MINISTRY OF SCIENCE AND TECHNOLOGY
MINISTRY OF EARTH SCIENCES
MINISTRY OF HEALTH & FAMILY WELFARE
GOVERNMENT OF INDIA

Certificate of Participation and Oral Presentation

This is to certify that Prof./Dr./Mrs./Mr./Ms. TAMALIKA PAUL of PRESIDENCY UNIVERSITY has participated and delivered an oral presentation entitled "HYDROXYCHAVICOL SENSITIZES IMATINIB-RESISTANT CHRONIC MYELOGENOUS LEUKEMIA CELLS TO TRAIL-INDUCED APOPTOSIS BY ROS-MEDIATED IAP DOWNREGULATION" under the theme "FRONTIERS AREA OF RESEARCH - LIFE SCIENCES" in the **Young Scientists' Conference** organized during **22-12-2020** to **24-12-2020** as a part of **India International Science Festival- 2020** by the Ministry of Science and Technology, Ministry of Earth Sciences, and Ministry of Health and Family Welfare, Govt. of India in collaboration with Vijnana Bharati (VIBHA).

Dr. Shekhar C. Mande
DG, CSIR & Secretary, DSIR

Dr. Vijay P. Bhatkar
President, Vijnana Bharati